XLIII. HYDROLYSIS OF PROTEINS WITH AN ALCOHOLIC SOLUTION OF HYDROGEN CHLORIDE.

PART I.

By CHARLES WEIZMANN and GANESH SAKHARAM AGASHE.

(*Received June 30th, 1913.*)

In the usual method of isolating the mono-amino-acids resulting from protein hydrolysis, aqueous hydrochloric acid is used as the hydrolysing agent, and subsequently a saturated alcoholic solution of hydrogen chloride is used for the purpose of esterification. An attempt was made to shorten this double process by using a saturated alcoholic solution of hydrogen chloride from the beginning to serve both as a hydrolysing and an esterifying agent.

Further, a saturated alcoholic solution of hydrogen chloride is undoubtedly a milder reagent than a saturated aqueous solution of the same, first, because the solubility of hydrogen chloride is smaller in alcohol than in water, and secondly, because it cannot be heated to as high a temperature as the aqueous solution; so it was expected that the process of hydrolysis would not be quite complete, but might stop at an earlier stage than that of the amino-acids.

While the research was in progress, Pribram [1911] and Abderhalden and Hanslian [1912] published their investigations, in which the action of this reagent on proteins was studied. But their object being quite different from that of the present investigation, they have not pursued the subject further.

In the present investigation, experiments have so far been made on caseinogen from cow's milk, and on silk-fibroin. In each case it was found that the protein was only partially attacked. The corresponding mono-amino-acids were isolated by the usual methods, and converted into copper salts, which were analysed. It must be admitted that the evidence of these analyses is not absolutely conclusive; but the authors believe that it is sufficient to show the identity of the various substances, which are known to be present in the different fractions by the more thorough investigations made by using the ordinary method.

Although the proportion of the protein attacked could not always be exactly determined, as the reaction liquid could not be filtered free from the solid floating in it, it appeared certain that a very large proportion had been attacked. The yield of the amino-acids, however, was comparatively poor. This makes it extremely probable that a large proportion of products, more complex than the simple amino-acids, had been formed. The isolation of individual substances from this complex mixture is an extremely difficult task; and experiments are still being carried on for that purpose.

Experimental.

Caseinogen.

200 grams of caseinogen were hydrolysed by boiling for several hours with 2 litres of alcohol, saturated with hydrogen chloride. There was some unchanged solid which could not be filtered. The mixture was, therefore, evaporated in vacuo, and the residue decomposed with sodium hydroxide, and extracted with ether in the usual way. A very strong smell of ammonia was evident throughout this process.

The ethereal extract was then fractionated under 14 mm. pressure, and the following fractions collected. The temperatures are those of the bath.

I –60°, II 60–100°, III 100–140°, IV 140–170°.

They were further worked up in the usual way.

Fraction I: This gave 0·4 gram of alanine, which was confirmed by analysing the copper salt.

0·0500 grm. gave 0·0162 grm. CuO. Cu = 25·92 per cent.
Calculated for alanine, Cu = 26·51 per cent.

Fraction II: This gave 3·5 grams of a mixture of leucine (also isoleucine), valine, and proline. They were isolated in the usual way, and confirmed by analysing the copper salts.

Leucine (and isoleucine):

0·1225 grm. gave 0·0293 grm. CuO. Cu = 19·13 per cent.
Calculated for leucine, Cu = 19·63 per cent.

Valine:

0·1098 grm. gave 0·0300 grm. CuO. Cu = 21·85 per cent.
Calculated for valine, Cu = 21·48 per cent.

Fraction III: This gave about 1·5 gram of a mixture of leucine, etc., and a little glutamic acid. The latter was converted into the copper salt, which was analysed.

0·0724 grm. gave 0·0280 grm. CuO. Cu = 30·93 per cent.
Calculated for glutamic acid, Cu = 30·45 per cent.

Proline: About 1 gram of this was obtained from the last two fractions. It was confirmed by analysing the copper salt, which was first made anhydrous.

0·0572 grm. gave 0·0154 grm. CuO. Cu = 21·53 per cent.
Calculated for proline, Cu = 21·77 per cent.

Fraction IV: Phenylalanine was isolated from this in the usual way, and confirmed by estimating the chlorine in the hydrochloride, of which about 0·4 gram was obtained.

0·1105 grm. gave 0·0778 grm. AgCl. Cl = 17·38 per cent.
Calculated for phenylalanine hydrochloride, Cl = 17·61 per cent.

The rest of the fraction gave 0·5 gram of a mixture of solids.

The dark brown residue in the distilling flask was extracted with hot water. On evaporating the aqueous solution, about 10 grams of a yellowish white powder were obtained, which was most probably a mixture of diketo-piperazines.

The residue in the extraction flask was not worked up as usual for re-esterification, but is being worked up with the object of isolating the more complex products of hydrolysis.

Silk-fibroin.

50 grams of silk-fibroin were boiled for several hours with about a litre of alcohol, saturated with hydrogen chloride. The fibre was entirely broken down, but a very fine solid was still floating in the brown liquid, which, in this case, could be easily filtered off. The fine solid weighed about 14 grams on drying.

The liquid was further dealt with exactly as in the last case; here, too, the smell of ammonia was very evident during the process of extraction with ether. The ethereal extract was distilled in the usual way, the temperature of the bath being only taken up to 100°. About 5·5 grams of a mixture of glycine and alanine were obtained. They were not separated quantitatively; but the picrate of glycine was prepared according to Levene's method, and melted at 190°.

No crystalline solid could be obtained from the residue in the distilling flask, which, however, gave very beautifully Denigès' test for tyrosine, as modified by Mörner [1902].

The residue from the extraction is being worked up for the isolation of complex hydrolytic products, as in the last case.

Another interesting experiment was made with silk-fibroin. About a gram of it was heated with about 10 c.c. of absolute alcohol saturated with hydrogen chloride, in a sealed tube for about seven hours at 110–120°. The whole of the fibroin was found to have gone into solution without any residue.

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XLIV. THE FLOWER PIGMENTS OF *ANTIRRHINUM MAJUS*. 2. THE PALE YELLOW OR IVORY PIGMENT.

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(*Received July 22nd, 1913.*)

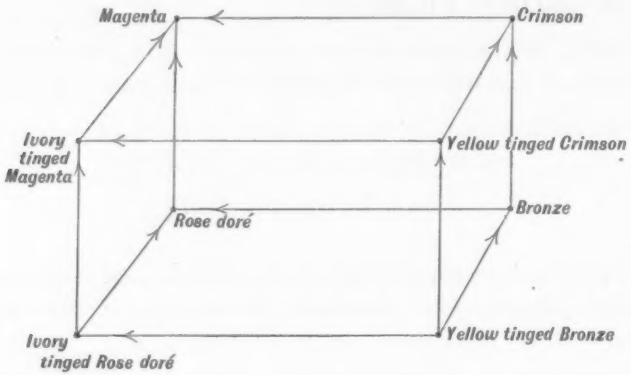
In a previous communication [Wheldale, 1913], the method of preparation of the crude pigment has been described. The varieties of *A. Majus* originally used for Mendelian experiments were the following:

White
Yellow
Ivory
Yellow tinged with bronze
Ivory tinged with rose doré
Bronze
Rose doré
Yellow tinged with crimson
Ivory tinged with magenta
Crimson
Magenta.

Ivory is dominant to yellow and since white may carry any factor but the one producing yellow, the result of crossing any variety with white depends on the gametic constitution of the white individual used. Of the varieties forming anthocyanin, the Mendelian relationship may be represented in the scheme on p. 442. Dominance is denoted by the direction of the arrows; thus rose doré is dominant to bronze, magenta to rose doré, etc.

It has been previously suggested that ivory contains a chromogen of the nature of a flavone, from which the red and purple anthocyanins are formed by stages of oxidation or polymerisation or both. Also that the pigment

of the yellow variety and of the yellow patch on the palate of all varieties (except white) is due to a second, more deeply coloured flavone. Microscopic examination and microchemical tests show that anthocyanin and yellow pigments are mostly limited to the epidermis of the corolla, while the inner tissues contain the ivory chromogen. It is obvious therefore that all crude extracts of entire flowers will contain two or more pigments.



Although no analyses have yet been made of the anthocyanins from crimson and bronze, yet purification of the crude pigment indicates that these colours are merely due to mixture of magenta with yellow, and red with yellow respectively and not to specifically different pigments.

The constituent pigments of the varieties may be thus expressed:

Yellow (ivory, yellow).

Ivory, lower lip (ivory, yellow).

„ upper lip (ivory).

Yellow tinged bronze, bronze, ivory tinged rose doré, rose doré (yellow, ivory and red).

Yellow tinged crimson, crimson, ivory tinged magenta (yellow, ivory, magenta).

Magenta, lower lip (yellow, ivory, magenta).

„ upper lip (ivory, magenta).

The crude pigment from yellow, ivory (upper lip), ivory tinged magenta, bronze, rose doré, crimson and magenta (both upper and lower lips separately) was extracted with ether for several months.

The yellow ether extracts were in each case crystallised from alcohol, the first deposits, consisting mainly of the ivory pigment, separated off and the

melting points determined. All the products melted between 336° and 340°. In five cases the acetyl derivative was prepared by boiling with acetic anhydride as described in the previous paper and crystallising several times from ethyl acetate. The final products were white substances crystallising in needles and all melting at 181–182°.

The five acetyl derivatives were submitted to combustion, giving the following results:

Acetyl derivations of ivory pigment from

		C.	H.
(1)	yellow	63·10 %	3·94 %
(2)	ivory, upper lip	62·84 %	3·99 %
(3)	red	63·61 %	4·14 %
(4)	bronze	63·10 %	3·92 %
(5)	ivory, tinged magenta	63·23 %	3·88 %

which agree closely with combustion results of the acetyl derivative previously obtained from ivory extracted from magneta [Wheldale, 1913]:

Acetyl derivative of ivory pigment from magenta:

	C.	H.
(i)	63·19 %	4·30
(ii)	63·21 %	4·22
(iii)	62·95 %	4·31

It was there suggested that the ivory pigment might be apigenin, of which the acetyl derivative should theoretically give:

C.	H.
63·64 %	4·04 %.

An acetyl derivative of apigenin was obtained by Kostanecki [Rupe, 1909]. Perkin [1897], on the other hand, was unable to acetylate apigenin but prepared a benzoyl derivative by the Schotten-Baumann process. The derivative thus prepared was slightly soluble in alcohol, more readily in benzene, from which it crystallised in colourless needles melting at 210–212°.

A benzoyl derivative having the same properties and melting point as the above was prepared from the ivory pigment by the following method. The pigment was dissolved in about five times its weight of pyridine, cooled in ice and benzoyl chloride added. On addition of dilute sulphuric acid, a yellow oil separated out which was collected and spread on a porous plate. It solidified after a time and was extracted with ether which removed benzoic acid and some yellow products leaving a white wax-like substance. The latter was crystallised from benzene. Melting point 210–212°.

Analysis results:

Benzoyl derivative of ivory pigment:

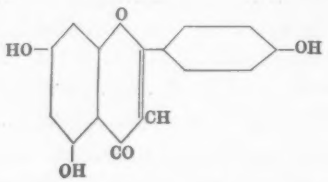
C.	H.
73·82 %	4·02 %.

Theoretical result for benzoyl derivative of apigenin:

C.	H.
74·23 %	3·78 %.

The properties of apigenin given by Aberhalden [1911] are as follows:

Formula, $C_{15}H_{10}O_5$.



M. Pt. 347°. Crystallises in yellowish-white platelets, readily soluble in alcohol, slightly in ether and hot water. Becomes bright yellow in alkali solution. The yellow solution in concentrated sulphuric acid has at first a greenish, later a bluish fluorescence. The alcoholic solution gives a blackish-brown coloration with ferric chloride and brown-red with ferrous sulphate.

The ivory pigment corresponds entirely with this description. From the acetyl derivative, the pure pigment was obtained by hydrolysis with alcoholic soda. On neutralisation with acid, the pigment came down as a yellowish-white precipitate and after crystallisation from dilute alcohol, melted at 347°.

We therefore conclude that the ivory pigment is apigenin, and that it is present in each of the main classes of varieties of *Antirrhinum* with the exception of the white. In the plant, apigenin exists undoubtedly as a glucoside, though the kind of sugar and the number of molecules attached still remain to be ascertained.

It appears possible that the deeper yellow pigment may prove to be a flavone, similar in constitution to apigenin, but deeper in colour owing to the presence of an additional hydroxyl group.

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XLV. OBSERVATIONS ON THE USE OF THE FOLIN METHOD FOR THE ESTIMATION OF CREATINE AND CREATININE.

By WILLIAM HENRY THOMPSON,
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(*Received July 22nd, 1913.*)

During a continuous use, for over three years, of the Folin colorimetric method of estimating creatine and creatinine we have made observations by way of testing its accuracy and limitations which we think may be useful to others if published. Some of these have meanwhile been pointed out by workers in other laboratories, but we include them for the sake of corroboration.

Procedure.

The colorimeter we mostly used was that of Jobin—a modification of the Du Boscq instrument. We also compared its readings with those of two similar instruments made by Pellin[2] and with one of the Kagenaar pattern as described by Hoogenhuyze and Verploegh [1905]. We found that consistent and reliable readings were obtainable with all, after a little experience in their use had been acquired.

For making the standard bichromate solution we used Kahlbaum's purest bichromate of potassium certified to contain 99·97 % of the pure salt. This was fused, then cooled in a desiccator and a semi-normal solution made from it containing 25·54 g. in the litre.

The readings of the colorimeter were made by one of us (W. H. T.), but

[1] Immediately before sending this paper for publication we learned, to our great sorrow, of the death of our co-worker, Thomas Arthur Wallace, at Agra in India, whither he had gone, a short while before, to take up a post in the Missionary College there. W. H. T., H. R. S. C.

[2] One of these was very kindly lent to us by Dr Ritchie of the Royal College of Physicians Laboratories, Edinburgh, to whom we express our best thanks.

were checked in all important cases by comparative readings made mostly by a second person in the laboratory familiar with the use of the instrument[1].

To avoid unconscious bias, the solutions in all the critical readings were made up by a second person and given to the reader under a number which gave no indication of which solution it was, this being disclosed only when the series was finished. We found this a very useful safeguard.

Great care was taken to obtain equal illumination of the two halves of the colorimeter. One important cause of inequality was found to be uneven blackening of the interior of the metal box in which the glass parallelopipeds are contained. The possibility of shifting of the zero on one or other side of the instrument was constantly examined and allowed for.

Another routine precaution, taken at the beginning of every set of readings and repeated as a rule after every four readings (if the series exceeded six in number), was to read the standard solution against itself. This we regard as of the utmost importance.

Few will believe, without actual experience, how much the reading of the standard by an individual may vary and how necessary is this control. The procedure also furnishes a ready test of fatigue, whether retinal or cerebral. When fresh, there is no difficulty in obtaining similar readings on the two sides using the standard solution in both tubes. When fatigued it was found that the readings were too high or too low, oftenest the latter and frequently to the extent of 0·5 mm.

The degree of illumination unquestionably affects the result. This has been pointed out by several workers. On dull days the readings tended to be lower than on bright days. Our instrument was placed opposite a large window, with northern aspect, and the readings made, for the most part, in the middle hours of the day. The practice of reading the standard against itself reduces the error for variation in the illumination.

Control of the Standard Solution.

Folin [1904], in fixing his bichromate standard, tested it against pure creatinine prepared from urine, also against creatinine solutions made by the conversion of creatine, and likewise against the double picrate of creatinine and potassium obtained from urine[2].

Of subsequent workers, there appear to be only three who controlled

[1] We are grateful to Professor Noel Paton and Dr Rosenheim for assisting us in this way on more than one occasion.

[2] This last was overlooked by one of us (W. H. T.) in a recent note on the subject [1913].

their standard solutions by the first of these procedures, namely: Hoogenhuyze and Verploegh [1905], Koch [1905] and Klercker [1907].

The creatinine used by Hoogenhuyze and Verploegh was prepared from urine and gave almost theoretical readings (8·14 mm. instead of 8·1 mm.). That of Koch was obtained from Merck and required 11·46 mgms. to give the standard reading of 8·1 mm. in the colorimeter, this being normally given by 10 mgms. of creatinine treated in the way described by Folin. Klercker used a solution of creatinine prepared from urine. The content of this was determined by estimating the total nitrogen and found to be 96·6 % of the theoretical, assuming all the nitrogen to be present as creatinine.

We endeavoured to control our standards by this method, but early attempts to prepare pure creatinine did not prove satisfactory. Later we prepared a small sample of pure creatinine free from creatine. With this we obtained theoretical readings. The difficulty we encountered of preparing pure creatinine is admitted by Folin. This has been largely reduced by his newer methods.

Of these latter, that of heating pure crystalline creatine in the autoclave at a pressure of 4·5 kilos per sq. cm. for 3 hours was tried by one of us. The yield was not so high as claimed for it by Folin, nor the creatinine so pure as anticipated, but the method proved valuable in giving an easy means of preparing pure creatinine picrate which we used later as a control.

Most workers have tested their bichromate standard solution against solutions of creatinine prepared by the conversion of pure creatine. This was the method followed by Dorner [1907], Gottlieb and Stangassinger [1907], Hoogenhuyze and Verploegh [1908], Weber [1908], Benedict and Myers [1907]. We gave it an exhaustive trial. For this purpose a large number of samples of pure creatine, obtained from different kinds of flesh (horse, rabbit, dog), were prepared from time to time, and purified by recrystallising six to eight times till absolutely pure. Several Dumas analyses of these were made, the results of which are here shown.

TABLE I. *Showing results of Dumas analyses of samples of creatine.*

	Creatine taken	N. evolved	Temp.	Pressure	N. per cent.	Theory
1	0·1056	29·4 c.c.	16° C.	740 mm.	32·21	32·06
2	0·2220	61·8	18	740·8	32·03	,,
3	0·1625	44·0	17	755·5	31·92	,,
4	0·2020	54·8	16·5	756	32·22	,,
5	0·1260	35·4	22	753·4	32·10	,,
6	0·2160	58·0	18·8	754·6	32·30	,,

(Two of these estimates, Nos. 5 and 6, were kindly made for us by W. Caldwell, M.A., Senior Demonstrator of Physiology in this school, who

also checked readings for us at various times. For this assistance we express to him our best thanks.)

There can be no doubt from the above analyses that our preparations of creatine were absolutely pure. This is borne out also by the fact that the crystals in every case, after being fully air dried at room temperature, lost the calculated amount of water of crystallisation when further dried in the oven at 110° C. In this latter drying—notwithstanding statements to the contrary—creatine does not decompose and we found it unsafe to trust for complete drying to a temperature lower than this. In weighing the anhydrous creatine, which is very hygroscopic, great care was taken to guard against the absorption of water.

In making the test solutions of creatinine we usually took the quantity of creatine requisite to give when fully converted a 0·1 % solution of creatinine, that is to say, 0·116 grm. of anhydrous creatine or 0·132 grm. of crystalline creatine for 100 c.c. In a few cases, solutions of anhydrous creatine of other strengths were used.

From a large number of estimates made with these solutions we found, as is shown in detail in Tables XIII and XIV, that the average conversion by the water bath method amounts to 96·5 % of the theoretical yield—varying from 93·1 % to 100·2 %. The readings on the colorimeter scale corresponding to these were 8·39 mm. for the average, 8·7 mm. for the lower, and 8·08 mm. for the higher, instead of 8·1 mm. for the theoretical yield.

On more carefully examining the results published by others we found that our experiences were not singular. Folin gives no data on this point in his first article, but speaks of the difficulty of complete conversion of creatine in the article in *Hammarsten's Festschrift* [1906]. Dorner [1907] with 0·1 % solutions of creatine obtained results varying from 85 to 100 % of the theoretical yield. Gottlieb and Stangassinger [1907] 83·19 to 99·98 (with 4·56 % HCl) and 91·55 % to 92·98 % (with 2·28 % HCl). Jaffé's [1906] best result with 2–2·5 % HCl was 94·3 %. It did not seem to us therefore that this method was sufficiently accurate to be used as a control for the standard bichromate solution and another test was substituted by one of us the results of which have been published [Thompson 1913]. It was found that solutions of creatinine picrate and also of the double picrate of creatinine and potassium, when taken in quantities equivalent to 0·1 grm. creatinine and treated in the usual way, fulfilled the required conditions, giving readings of 8·1 mm. in the Du Boscq colorimeter. This result settled some doubts which had previously existed in our minds with regard to the accuracy of the Folin standard.

Conditions which affect the Readings.

Our object was not to test all the possible conditions which might affect the results. We confined ourselves to those which came under our notice in the application of the method to our work.

These were (1) in the estimation of creatine, the best amount of acid (HCl) to use for the conversion into creatinine; (2) the optimum time and temperature for the development of the colour in the estimation of creatinine and creatine; (3) the influence of the quantity of alkali added for the same purpose; (4) the relative values of readings at different parts of the scale; (5) the influence of urinary pigment on the estimation of creatine in urine; (6) the estimation of creatine in the presence of dextrose and the use of phosphoric acid; (7) the recovery of creatine from diabetic urine; and (8) the reliability of the autoclave method.

1. *Estimation of Creatine. The amount of acid to be used in the conversion.*

For the conversion of creatine into creatinine, Folin in 1904 recommended 5 c.c. of normal HCl to be added to 10 c.c. of a dilute solution of creatine. Later [1906] he increased this amount to 10 c.c., a quantity which has been adopted by most subsequent workers. A few however, namely Dorner [1907], Hoogenhuyze and Verploegh [1905], have used double that quantity, namely 20 c.c. of N. HCl.

We have tested all three quantities and are convinced that the best results, both with the water bath and autoclave methods, are got by using 10 c.c. N. HCl with 10 c.c. of weak creatine solution. The conversion with 5 c.c. is liable to be less complete, particularly if glucose be present.

From several observations the following two consecutive sets of readings are taken:

N. HCl	Method	Reading	N. HCl	Method	Reading
20 c.c.	water bath	8·59 mm.	20 c.c.	autoclave	8·52 mm.
20	autoclave	8·69	10	,,	8·26
10	,,	8·46	5	,,	8·32
5	,,	8·6			

The readings with 20 c.c. and 5 c.c. are uniformly higher, that is, the yield is uniformly less, than with 10 c.c. It would seem as if a destruction of creatinine took place when 20 c.c. of acid were used.

Except at the very outset we have always used in these investigations 10 c.c. of N. HCl for the conversion of creatine.

2. *The Time and Temperature for Development of the Colour.*

Folin originally gave a time limit of 5 to 10 mins. for the development of the Jaffé reaction. Later [1906] he restricts the time to 5 mins. and this period has been adopted by most workers. Some however have departed from it, thus Dorner [1907] gave 5–15 mins., Benedict and Myers [1907] only 3·5 mins., Mellanby [1908] 5 mins. with some latitude, Mendel and Rose [1911] 10 mins.

Comparatively few observers however have recorded the temperature which they adopted. Amongst these are Hoogenhuyze and Verploegh [1905], who diluted with water at 15° C., Dorner [1907], who cooled the solution after adding the picric acid and Klercker [1907], who used water for dilution at different seasons of the year of about the same temperature. Mellanby [1908] also states that it is necessary for the temperature to be constant but does not give the degree.

We found with temperatures of 10° C. to 15° C. that the maximum colour was not developed under eight minutes. Between 15° C. and 17° C. the time required was seven minutes, above 17° C. and below 20° C. five minutes is sufficient.

The results are shown in the following table:

TABLE II. *Creatinine estimation: influence of time and temperature on the development of the Jaffé reaction.*

Readings Time 5 mins.	Readings Time 7 mins.	Temp.	Method of conversion
8·75 mm.	8·52 mm.	10	Water bath 5 hours.
9·30	8·85	10	Autoclave HCl 25 mins.
8·55	8·45	15	Water bath.
8·76	8·50	15	Autocl. HCl 25 mins.
8·80	8·70	15	Autocl. H_3PO_4 30 mins.
8·30	8·11	15	Water bath HCl.
8·48	8·35	17	Water bath HCl 3 hours.
8·29	8·30	20	Water bath HCl 3 hours.

Nearly all our observations were made at a temperature lying between 15° C.–17° C. The flasks in which the reaction was carried out were weighted with leaden rings and kept in a basin of water at this temperature.

3. *Effect of the amount of alkali used in the Folin method.*

We found that the quantity of alkali added to develop the colour of the Jaffé reaction distinctly affected the results. For creatinine Folin recommended 5 c.c. of 10% NaOH solution; for creatine, the same amount over and above

that necessary to neutralise the acid used in the conversion. These quantities have been adhered to generally by others with few exceptions. Thus Benedict and Myers [1907] used 10 c.c. for 3·5 mins.; Mellanby [1908] concluded that no great accuracy was necessary; he got the same results with 10 c.c. and 3 c.c. His observations were chiefly concerned with meat extracts. Grindley and Woods [1906], also working with meat extracts, used 5 c.c. to 10 c.c. 10% NaOH. Hehner [1907] soon afterwards stated, in a short publication giving no data, that an excessive quantity of alkali diminished the colour in the case of meat extracts, and that to obtain the best results the amount of alkali added must be quite small. He also considered that more picric acid than the quantity prescribed by Folin should be used when dealing with these extracts. In reply to this Emmett and Grindley [1907] repeated the work of Grindley and Woods, finding that in the determination of preformed creatinine the use of a small or large amount of alkali made almost no difference, but that slightly better results were obtained with 10 c.c. or 15 c.c. than with 5 c.c. For transformed creatine 10 c.c. and 15 c.c. gave the same results, and both were better than 5 c.c. It is not clear, however, that the acid used in the conversion was neutralised before adding the picric acid solution, nor was their method strictly comparable to that followed by others—the quantities of creatinine containing solution were much larger.

Cook [1909] also found in estimating the creatine content of meat extracts, that 5 c.c. of alkali did not give the maximum colour, while 10 c.c. and 15 c.c. gave identical results. With solutions of creatinine he found that 5 c.c. and 10 c.c. gave similar results. Accordingly, he recommended the use of 10 c.c. all round. For a considerable time we followed this recommendation without question, but later discovered that 10 c.c. of 10% NaOH destroyed some of the creatinine and gave too low results. This applies chiefly to pure solutions of creatinine but also, though to a less extent, to urine. We have not tested the point in the case of meat extracts. Our results are shown in the following readings.

TABLE III. *Influence of the quantity of alkali on the colour developed.*

	Readings with 5 c.c. NaOH of 10% strength		Readings with 10 c.c. NaOH of 10% strength
	(*a*) Creatinine in aqueous solution		
	8·29		8·75
	8·25		8·70
	8·58		8·75
	8·78		9·05
	8·20		8·58
Mean	8·42	Mean	8·77

TABLE III (*continued*)

	(*b*) Creatinine in urine		
	9·54		9·66
	9·05		9·45
	9·55		9·75
	6·30		6·50
	7·51		7·9
	8·25		8·27
	6·26		6·41
	5·73		5·85
Mean	7·77	Mean	7·97

It will be seen that with pure solutions of creatinine, the average reading of the series, when 5 c.c. of alkali were used, was 8·42 mm. (= 0·0966 % of creatinine): when 10 c.c. of alkali were used the average reading was 0·35 mm. higher, that is 8·77 (= 0·0924 % of creatinine). There was also a difference with urine though less marked. The mean of the series of readings with 5 c.c. of alkali is 7·77 mm. (= 0·1042 %), that with 10 c.c. alkali is 7·97 mm. (= 0·1016 % creatinine). So far as these solutions are concerned we are unable to confirm Cook: on the contrary, we find that better results are given when Folin's original directions are strictly followed.

4. *Relative values of readings at different parts of the scale.*

It has been generally accepted that within a lower limit of 5 mm. and an upper one of 12 mm., as stated by Folin, the values of the readings are proportional. We have not found this to be so, nor is it generally true in colorimetry that the dilution ratio of a solution is inversely proportional to the height of the column of coloured solution. Folin naturally refers to this, and explains the exceptional result in his method as follows. He concludes that dilution produces a diminution of the total colour of the solution—not an increase such as would arise if the colour were due to a red ion—but that the diminution is hidden by the increased relative depth of tint which occurs with increase of the height of column seen through. It has, however, since been shown by Chapman [1909] that the colour in the Jaffé reaction is due not alone to the formation of picramic acid, as had long been held, but to a mixture of picramic acid and a still redder reduction product of picric acid, namely diaminonitrophenol. It is doubtful therefore if Folin's explanation will hold good, nor are the facts quite in accordance with his view.

On this point we made a large number of observations, comparing in each case the reading given at a certain dilution with that given by the same solution diluted to one and a quarter, one and a half, or double the volume.

In the following table we give some of our results confining these to a comparison of readings with dilutions to 250 c.c. and 500 c.c., but making the selection so as to show the results with different solvents. These latter were water, dog's urine, normal human urine, and diabetic human urine.

TABLE IV. *Relative value of readings at different parts of colorimeter scale.*

	Solvent	Readings Dilution 10–250	Readings Dilution 10–500	Ratio	
1.	Water (HCl)	6·3 mm.	11·2 mm.	1 : 1·75	
2.	,,	6·5	11·36	1 : 1·72	
3.	,,	6·55	11·60	1 : 1·77	
4.	,,	5·50	10·10	1 : 1·80	
5.	,,	5·45	9·80	1 : 1·77	
6.	,,	6·15	11·00	1 : 1·79	Mean = 1 : 1·76
7.	,,	6·28	11·00	1 : 1·75	
8.	,,	6·15	10·90	1 : 1·77	
9.	,,	6·15	10·70	1 : 1·74	
10.	,,	6·30	10·90	1 : 1·73	
11.	,,	6·24	11·15	1 : 1·78	
12.	Dog's urine (HCl)	4·80	8·90	1 : 1·85	
13.	,, ,,	4·43	8·50	1 : 1·92	
14.	,, ,,	5·90	11·30	1 : 1·91	
15.	,, ,,	4·85	9·30	1 : 1·92	Mean = 1 : 1·91
16.	,, ,,	4·63	9·00	1 : 1·94	
17.	,, ,,	4·60	8·96	1 : 1·95	
18.	,, ,,	4·87	9·24	1 : 1·90	
19.	Human urine (HCl)	6·30	12·20	1 : 1·94	
20.	,, ,,	4·30	8·10	1 : 1·88	
21.	,, ,,	5·55	10·30	1 : 1·87	Mean = 1 : 1·91
22.	,, ,,	5·50	10·67	1 : 1·94	
23.	Diabetic urine	5·22	10·10	1 : 1·93	
24.	,, ,,	6·60	12·80	1 : 1·94	
25.	,, ,,	6·60	12·00	1 : 1·82	Mean = 1 : 1·87
26.	,, ,,	6·25	11·30	1 : 1·81	

On examining the list it will be seen that the higher reading is never double the lower and that the ratio differs considerably when urine is compared with water as the solvent, also that there is a difference, though not marked, between normal and diabetic human urine. On the other hand, the dilution ratio is identical for human and dog's urine. The mean ratio instead of being 1 to 2, is, for solutions in water 1 to 1·76, in dog's urine and human urine 1 to 1·91, in diabetic urine 1 to 1·87. These results were borne out by dilutions to other degrees than one to two.

Our general conclusions are that for accurate work readings can only be regarded as strictly proportional if they lie between a lower limit of 7 mm. and an upper one of 9 mm. If separated by a wider interval a correction factor has to be applied, and this varies for different solvents, but for all

practical physiological purposes may be based on the mean of all the above ratios for a difference of column of 5 mm., viz. 1:1·8 instead of 1:2. In general therefore the upper readings of the scale give relatively too high results, the lower readings on the contrary too low results, as compared with the normal reading of 8·1 mm.

5. *The influence of urinary pigment: recovery of creatine from normal urine.*

Several observers have called attention to the influence of urinary pigment. Weber [1908] found that the darkening which occurs on boiling urine with HCl lowers the reading and increases the estimation of creatine by fully 5%. Benedict [1912] also found a similar effect and recommended the use of granulated zinc in the boiling to remedy it. Rose [1912] did not find this procedure efficacious with diabetic urines and recommended the use of phosphoric acid instead of hydrochloric. Dreibholz [1908], with diabetic urine, found a difficulty in matching the colour with the standard bichromate solution after boiling the urine with hydrochloric acid, but failed to find a useful remedy. With normal urine when the pigment was removed by filtration the result was not affected.

Early in these investigations it was clear to us that in the case of dog's urine the darkening on boiling with hydrochloric acid lowered the colorimeter readings. We therefore proceeded to investigate the point, and did so in two ways, both of which were applied to normal human and dog's urine.

In the first method the ordinary creatine readings of a series of urines were compared with those given by similar samples of the same urines after boiling with an equal quantity of normal HCl. In the boiling we used not alone the water bath for three hours, but also the autoclave at 117°–120° C. for two different periods, namely, 15 mins. and 25–30 mins. The results are shown in the following tables:

TABLE V. *Showing colorimeter readings of human urine before and after boiling with normal HCl.*

	Unboiled	Water bath 3 hours	Autoclave 15 mins.	Autoclave 25–30 mins.
1.	7·8	7·53	7·5	7·6
2.	6·5	6·3	6·5	6·51
3.	8·1	7·9	8·15	8·1
4.	7·24	7·15	7·16	7·26
5.	7·6	7·3	—	7·5
6.	6·25	6·16	—	6·1
7.	7·51	7·54	—	7·5
Mean	7·29	7·13	7·19	7·22

The effect on human urine is therefore very slight. Taking the mean yield of the unboiled urines as 100 %, that of the water bath series amounts to 102·25 %, and of the autoclave, 25–30 mins., to 101 %. The yield of those boiled in the autoclave for 15 mins., when compared with the same group of urines unboiled, works out at 101·1 %.

TABLE VI. *Showing similar readings to the above for dog's urine.*

	Unboiled	Water bath 3 hours	Autoclave 15 mins.	Autoclave 25–30 mins.
1.	6·2	5·7	6·35	6·06
2.	7·4	6·08	6·46	6·45
3.	7·1	6·4	6·84	6·73
4.	7·74	7·3	7·54	7·47
5.	6·62	6·2	—	6·67
6.	8·5	7·87	—	7·9
7.	9·3	8·03	—	8·13
Mean	7·55	6·8	6·8	7·06

The results for dog's urine, taking the mean of the unboiled series as 100 %, work out as 111 % for urine treated by the water bath method, and 106·9 % for the same boiled in the autoclave for 25–30 mins. The mean yield of the smaller autoclave series boiled for 15 mins. gives 104·6 % when compared with that of the same group unboiled. It is interesting to note that in both series the effect on the pigment is more pronounced in the water bath than in the autoclave method.

The second method consisted in comparing the recovery of creatine when dissolved in urine with that of the same substance dissolved in water. The procedure adopted in testing the recovery of creatine from urine was as follows. Normal urines were taken and known quantities of the pure samples of creatine we used throughout this research were dissolved in them—for the most part 0·116 g. in 100 c.c. (= 0·1 % of creatinine).

Determinations were then made, (1) of the preformed creatinine in the urines unboiled, (2) of the readings of the same urines without any addition of creatine but treated by the water bath and autoclave methods, (3) of the total creatinine in the urines after creatine was added, these being treated as in (2). Two sets of subtractions were then made; (*a*) the creatinine of the unboiled urine was subtracted from the total creatinine of the urine to which creatine was added; (*b*) the creatinine of the boiled urine was subtracted from that of the same urine plus creatine.

The values for the first set of deductions should give too high results for recovered creatine, at all events in the dog's urine, if the darkening of the pigment affect the recovery. Those of the second set, presumably the

true creatine values, should correspond to the results of recovery of creatine from solution in water provided no other disturbing factor, than the effect of the pigment, entered into the reaction.

Observations were made both with human urine and with that of the dog. The following tables show the results:

TABLE VII. *Showing the recovery of creatine from dog's urine: expressed as volume percentages of the urine taken.*

	Creatine added expressed as creatinine	Creatine recovered expressed as creatinine water bath 3 hrs.		Creatine recovered expressed as creatinine autoclave 25 mins.	
		(*a*)	(*b*)	(*a*)	(*b*)
1.	0·05	0·0627	0·0598	0·0575	0·0567
2.	0·05	0·0594	0·0475	0·0613	0·0533
3.	0·10	0·1003	0·1003	0·0916	0·0910
4.	0·10	0·1029	0·0935	0·0939	0·0892
5.	0·10	0·0892	0·0809	0·0925	0·0930
6.	0·10	0·1297	0·1221	0·1022	0·0950
7.	0·05	0·0598	0·0461	0·0605	0·0478
Totals	0·5500	0·6040	0·5502	0·5595	0·5260
		=109·8 %	=100 %	=101·7 %	=95·4 %

The total yield of the first set of values by the water bath method, column (*a*), works out as 109·8 % of the amount added. That obtained by the second method of deducting, column (*b*), gives a yield of 100 %. The corresponding values furnished by the autoclave method (25 mins. at 117–120° C.) are 101·7 % and 95·4 % respectively. Those therefore in column (*a*) show an increase due to pigment of 10 % by the water bath method and of 6–7 % by the autoclave method, figures which correspond very closely with the effects of darkening of the pigment of dog's urine seen in Table VI. Moreover, the mean of the recovery by the two methods, water bath and autoclave, obtained by the second method of deduction is 97·7 % which is very close to the average recovery from solution in water mentioned in the earlier part of this paper, namely, 96·5 %.

The results of recovery from human urine are seen in Table VIII.

On examining these figures it will be seen that the results of column (*a*) in the water bath method, which include the pigment effect, are just over 2 % higher than those of column (*b*). Similarly, those of the autoclave method show a difference of 1·2 % due to the pigment, and these differences correspond almost exactly with those given earlier for the effect of the pigment in human urine alone. The mean result for the two methods (water bath and autoclave), taking column (*b*) in each case, which excludes

the pigment effect, is 98·26 %, that is slightly higher than the general average for aqueous solutions. It may be concluded therefore that the darkening of the pigment is the only disturbing factor in normal urine in the recovery of creatine, and that its augmenting effect in round numbers is 2 % for human urine and 10 % for dog's urine. The effect, moreover, may be practically eliminated by the procedure of boiling the urine for the normal period (in any given research) and deducting the results from those given by the urine of subsequent periods treated in identically the same way—that is assuming the pigment has remained unaltered throughout.

TABLE VIII. *Showing the recovery of creatine from human urine: expressed as volume percentages of the urine taken.*

	Creatine added expressed as creatinine	Creatine recovered expressed as creatinine water bath 3 hrs.		Creatine recovered expressed as creatinine autoclave 25 mins.	
		(*a*)	(*b*)	(*a*)	(*b*)
1.	0·05	0·0521	0·0496	0·0563	0·0563
2.	0·10	0·0980	0·0970	0·0970	0·0974
3.	0·10	0·0980	0·0937	0·0958	0·0944
4.	0·10	0·1111	0·1092	0·1104	0·1072
5.	0·10	0·0881	0·0885	0·0922	0·0920
Totals	0·45	0·4473 =99·4 %	0·4380 =97·33 %	0·4517 =100·4 %	0·4473 =99·2 %

6. *Estimation of creatine in the presence of dextrose.*

Different statements had been made with regard to the influence of dextrose on the Jaffé reaction in its application to the estimation of creatinine and creatine. Klercker [1907] found that on long standing, when glucose was present, the colour became deeper; Hoogenhuyze and Verploegh [1905] that glucose produces no effect in the time necessary for the Folin estimation; Dreibholz [1908] that the caramel produced on boiling diabetic urine with HCl adversely affected the estimation of creatine; Taylor [1911] that glucose introduces no effect on the estimation of creatine unless present to above 5 %; while Rose [1912] strongly supports the view of Dreibholz.

We felt it necessary from the outset of our investigations to ascertain for our own satisfaction which statement was to be accepted. Our first observations dealt with aqueous solutions of creatine and dextrose, later we extended them to diabetic urine in which creatine was dissolved.

TABLE IX. *Estimation of creatine in presence of dextrose.*

	Water bath 3 hours HCl	Autoclave 25 mins. HCl	Autoclave 30 mins. H_3PO_4 3 %	Autoclave 30 mins. H_3PO_4 2 %	Dextrose present	Theoretical amt. expressed as creatinine
1.	0·1012	0·1020	—	—	5 %	0·1000
2.	0·1004	0·1002	—	—	10 %	,,
3.	0·0937	0·0936	0·0886	—	10 %	,,
4.	0·0910	0·0907	0·0814	—	10 %	,,
5.	0·0895	0·0900	0·0815	0·0900	10 %	0·0900
6.	0·0975	0·0968	0·0917	0·0960	10 %	0·1000
7.	0·0324	0·0366	0·0323	0·0326	10 %	0·0342
8.	0·0960	0·0964	0·0850	0·0889	10 %	0·1000
Av. % of recovery	96·6	96·6	86·7	92·5		0·1000

The foregoing table gives the first series of observations. It includes also a comparison of the results of the water bath method with those of the autoclave. Further, the efficacy of phosphoric acid in strengths of 3 % and 2 % for the recovery of creatine is compared with that of normal hydrochloric acid.

On examining the table it will be seen that with HCl—both water bath and autoclave methods—the average percentage of recovery (96·6 %) is practically the same as when dextrose is not present in the solution. This, as previously mentioned, was 96·5 %. Our conclusion, therefore, is that dextrose *per se* has very little, if any, influence on the results within the time necessary for estimating creatine.

In the above observations we used 10 c.c. of normal HCl for 10 c.c. of creatine solution, but in some of our early work the quantity of acid added was only 5 c.c. In these we found, when the quantity of sugar was 5 % or over, that the creatine results were much too low. This has also been the experience of M. Ross Taylor [1911]. It is essential therefore to use the larger quantity of acid.

It has been stated by Rose [1912] that the disturbing influence of dextrose is due to the action of hydrochloric acid in causing a transformation of some of the sugar into caramel. It occurred to him therefore to employ phosphoric acid in the conversion of creatine when dextrose is present, since it does not caramelise sugar. Rose claims to have obtained better results in this way than with hydrochloric acid. We tested the effects of the two acids, using solutions of creatine both in the presence and absence of sugar. In these tests varying strengths of phosphoric acid were used, 5 %, 4 %, 3 % and 2 %. The results are shown in Table IX. In all cases the best results were obtained by us with 2 %, by Rose, however, with 3 % phosphoric acid, the

volume of acid added in both sets of observations being 20 c.c. The results with 5 % and 4 % phosphoric acid were much inferior—these strengths causing a considerable destruction of creatinine.

We then felt it necessary to make a comparison of these same acids, using creatine in aqueous solution. The following table gives the results:

TABLE X. *Estimation of creatine in aqueous solution: comparison of hydrochloric and phosphoric acid in transforming creatine into creatinine.*

	Water bath N. HCl	Autoclave 30 mins. H_3PO_4 3 %	Autoclave 30 mins. H_3PO_4 2 %	Theoretical amt. expressed as creatinine
1.	0·0942	0·0910	0·0940	0·1000
2.	0·0935	0·0885	0·0920	,,
3.	0·0931	0·0915	0·0921	,,
4.	0·0928	0·0910	0·0931	,,
5.	0·0924	—	0·0925	,,
6.	0·0951	0·0932	0·0944	,,
7.	0·0976	0·0931	0·0976	,,
8.	0·0972	0·0944	0·0980	,,
9.	0·0960	0·0935	0·0942	,,
Mean recovery %	94·6	81·8	94·6	,,

On examining the table it will be seen that the results with normal HCl and 2 % H_3PO_4 closely correspond, while those with 3 % H_3PO_4 are considerably lower. The mean result with HCl is 94·6 % of the theoretical yield; with 2 % phosphoric acid the same; with 3 % phosphoric acid 81·8 %. It still remained to test the recovery of creatine from diabetic urine—the results are shown in the following section.

7. *Recovery of creatine from diabetic urine.*

In these observations we made use of a series of diabetic urines obtained from time to time from clinical hospitals in Dublin through the kindness of members of the staff. We applied the same four methods for recovery of creatine from diabetic urine which were used when the substance was added to aqueous solutions of dextrose (see Table IX). The results are necessarily somewhat more complicated than with simple solutions, since the preformed creatinine and creatine had to be determined separately.

The full data are given in Table XI. Pure creatine was added to eight samples of diabetic urine obtained from different patients. Three of the samples contained both acetone and aceto-acetic acid, four were free from both these bodies, and one contained a trace of aceto-acetic acid but no acetone.

TABLE XI.

Showing the recovery of creatine when added to diabetic urines.

Urine contained	Preformed creatinine per cent.	Creatine added, expressed as creatinine	Preformed creatine of urine as found by (a) HCl W. B. method	(b) HCl autocl. 25 mins.	(c) H_3PO_4 3% autocl. 30 mins.	(d) H_3PO_4 2% autocl. 30 mins.	Creatine recovered as found by (a) HCl W. B.	(b) HCl autocl. 25 mins.	(c) H_3PO_4 3 %	(d) H_3PO_4 2 %
1. Dextrose 5·2 %. No acetone or aceto-acetic acid	0·0570	0·1000	0·0355	0·0346	0·0320	—	0·0948 = 94·8 %	0·0950 = 95 %	0·0945 = 94·5 %	—
2. Dextrose 10 %. Both acetone and aceto-acet. ac.	0·0389	0·1000	0·0311	0·0311	0·0310	—	0·0720 = 72 %	0·0720 = 72 %	0·0611 = 61·1 %	—
3. Dextrose 9·7 %. Both acetone and aceto-acet. ac.	0·0365	0·1000	0·0249	0·0319	0·0051	—	0·0859 = 85·9 %	0·8660 = 86·6 %	0·0812 = 81·2 %	—
4. Dextrose 4·6 %. No acetone or aceto-acetic acid	0·0467	0·1000	0·0033	0·0039	0·0015	0·0040	0·0946 = 94·6 %	0·0923 = 92·3 %	0·0931 = 93·1 %	0·0964 = 96·4 %
5. Dextrose 3·8 %. No acetone or aceto-acetic acid	0·0675	0·1000	0·0128	0·0129	0·0104	0·0044	0·1065 = 106·5 %	0·1053 = 105·3 %	0·1001 = 100·1 %	0·9250 = 92·5 %
6. Dextrose 7·7 %. Both acetone and aceto-acet. ac.	0·0960	0·1000	0·0445	0·0441	0·0213	0·0267	0·0889 = 88·9 %	0·0913 = 91·3 %	0·0872 = 87·2 %	0·0864 = 86·4 %
7. Dextrose 2·8 %. No acetone, trace of aceto-acetic acid	0·0475	0·1000	0·0021	0·0022	0·0010	0·0000	0·0968 = 96·8 %	0·0928 = 92·8 %	0·0860 = 86·0 %	0·0942 = 94·2 %
8. Dextrose 1·7 %. No acetone or aceto-acetic acid	0·0374	0·0843	0·0268	0·0269	0·0196	0·0249	0·0804 = 95·4 %	0·0786 = 93·24 %	0·0798 = 94·6 %	0·0749 = 88·8 %

On examining the results it will be seen that the percentage recoveries with the different methods were as follows: (1) water bath HCl, 72% to 106·5%, the mean of the series being 91·8%; (2) autoclave HCl, 72% to 105·3%, the mean being 91·2%; (3) autoclave H_3PO_4 3%, from 61% to 100%, the mean being 87·1%; (4) autoclave H_3PO_4 2%, from 86·4% to 96·4%, the mean being 91·76%.

The results where HCl has been used are about 5% inferior to the corresponding recoveries from aqueous and dextrose solutions. Those with 2% H_3PO_4 are less variable but the mean (taken however from a smaller series) is identical with that of HCl. With 3% H_3PO_4 the results are less favourable. We are forced therefore to conclude that while no special advantage is gained by using phosphoric acid, there is a liability to destruction of creatine by this reagent even with a strength of 3%. It will be remembered that the same effect was found in the recovery of creatine from aqueous solutions both in the presence and absence of glucose.

The remarkable feature, however, of the results is the variability in the degree of recovery from different diabetic urines. This was not seen in the recovery from normal urine nor from aqueous or dextrose solutions, and points to a disturbing factor other than pigment or sugar. It is also remarkable that the lowest results were obtained from urines containing acetone and aceto-acetic acid. We have not studied the effects of either of these bodies, but they have been called attention to by Jaffé (acetone), Folin (acetone, aceto-acetic acid, and aceto-acetic ester), Klercker (acetone), Hoogenhuyze and Verploegh (acetone), Krause [1910] (acetone and aceto-acetic acid), Wolf and Österberg [1911] (aceto-acetic ester), Rose [1912] (aceto-acetic acid), Greenwald [1913] (acetone and aceto-acetic acid). Krause and Greenwald find the disturbing effect of aceto-acetic acid more serious than that of acetone. Its presence increased the values for preformed creatinine, and thereby lowered those of creatine. This is in harmony with our observations, but we think the matter deserves a more thorough investigation, starting with solutions of pure creatine to which dextrose, acetone, and aceto-acetic acid are added in succession.

Since the foregoing was written Graham and Poulton [1913] have published a note in which they show that aceto-acetic acid and its sodium salt diminish the colour of the Jaffé reaction and thus lower the estimate of creatinine. Aceto-acetic ester, used by Wolf and Osterberg, also by Folin, has no appreciable effect.

8. *Comparison of the autoclave and water bath methods.*

It may seem superfluous to have undertaken this part of the work, but at the period when we began it, the reliability of the autoclave method was not universally accepted. We therefore decided to test it for ourselves, and also to determine the optimum time in the autoclave for the transformation of pure creatine into creatinine at the temperature (117° C. to 120° C.) recommended by Benedict and Myers.

Two series of observations were made. In the first the results of heating on the water bath for three hours were compared with those of the autoclave, heated for 15 mins., 25 mins., and 35 mins. respectively. They are given in Table XIII:

TABLE XIII. *Creatine estimation: comparison of water bath and autoclave methods.*

No.	Water bath 3 hrs.	Autoclave 15 mins.	Autoclave 25 mins.	Autoclave 35 mins.	Theoretical amt. expressed as creatinine
1.	0·1002	0·0994	0·1004	0·0997	0·1000
2.	0·0981	0·0944	0·0982	—	,,
3.	0·0976	0·0935	0·0982	0·0942	,,
4.	0·0997	0·0942	0·0995	—	,,
5.	0·1000	0·0947	0·1000	0·1006	,,
6.	0·0944	0·0880	0 0962	0·0953	,,
Total	0·5900 =98·35 %	0·5642 =94·03 %	0·5925 =98·78 %	0·3898 =97·45 %	0·6000

It will be seen that the results of heating in the autoclave for 15 mins. are too low, while those for 25 mins. are identical, or almost so, with the water bath results. Consequently we are of opinion that the optimum time in the autoclave is 25 mins. at a temperature of 117° C. The results of heating for 35 mins. are slightly lower than those for 25 mins., indicating that the optimum period has been exceeded.

The second series gives a number of comparisons between the water bath results and those of the autoclave heated for one period only, namely, 25 mins.

On examining the mean results of the foregoing a remarkably close correspondence—indeed identity—will be seen, though the degree of conversion 95·7 % is a little lower than the average as given at the beginning of this paper, namely 96·5 %, which was obtained by taking the gross mean of the determinations in Tables XIII and XIV. When we compare this

with the gross mean of the autoclave determinations (25 mins.) the correspondence also works out very close, namely 96·6 % as against 96·5 %. Observations were also made for periods of one hour and two hours in the autoclave. In both cases there was a considerable destruction of creatinine, the results at one hour giving a yield of 92·4 % and at two hours of 88·6 %.

TABLE XIV. *Creatine estimation: comparison of water bath and autoclave methods.*

No.	Water bath 3 hours	Autoclave 25 mins.	Theoretical amount expressed as creatinine
7.	0·0975	0·0969	0·1000
8.	0·0964	0·0961	,,
9.	0·0964	0·0961	,,
10.	0·0994	0·0999	,,
11.	0·0994	0·0993	,,
12.	0·0942	0·0948	,,
13.	0·0935	0·0932	,,
14.	0·0931	0·0927	,,
15.	0·0931	0·0925	,,
16.	0·0944	0·0971	,,
17.	0·0959	0·0953	,,
18.	0·0953	0·0942	,,
19.	0·0960	0·0958	,,
20.	0·0955	0·0959	,,
Total	1·3401 = 95·7 %	1·3398 = 95·7 %	1·4000

It may be stated that we found no difficulty in obtaining consistent and concordant results with the autoclave, provided that care was taken to see that the time and temperature were kept constant and that the autoclave was not opened till the pressure had fallen to zero. We allowed at least ten minutes for this and then opened the valve slowly. Having set the autoclave for 117° C. it is desirable if possible not to use it for other purposes necessitating different temperatures, during the time it is in use for estimating creatine. It must be borne in mind that the velocity of reaction is greatly accelerated as compared with that at 100° C. and therefore slight differences of time and temperature produce much greater effects.

We may perhaps add also in regard to the water bath that our ordinary rule was to immerse the flask in the water and use a reflux condenser. Latterly we have returned to the use of the funnel and watch glass for condensation and find it more convenient, but the flask should be immersed in the water. If placed on the top of the water bath the results are less consistent. Dr Rosenheim informed one of us (W. H. T.) that he had had the same experience.

Summary of Results.

1. For control of the bichromate standard solution used in the Folin method of estimating creatinine and creatine we did not find the degree of conversion of creatine into creatinine by boiling with normal HCl sufficiently constant. We recommend for this purpose creatinine picrate as described by Thompson.

2. For the estimation of creatine in weak solutions the best results were got by using an equal quantity of N. HCl and boiling either on the water bath for three hours or in the autoclave for 25 minutes at 117° C.

3. For the development of the colour in the Folin method the optimum time and temperature in our hands proved to be 7 mins. at 15°–17° C.

4. The addition of too much alkali reduces the colour. For aqueous solutions and urine the best results are given by the quantity recommended by Folin, namely 5 c.c. of 10 % NaOH over and above what may be necessary to neutralise the solution.

5. The range of proportional readings on the colorimeter scale should not go lower than 7 mm. nor higher than 9 mm. If it is necessary to compare readings separated by a wider interval a correction factor should be used as explained in the body of this paper.

6. The darkening of the pigments of urine which occurs on boiling with normal HCl adds to the estimate of creatine-creatinine contained in it. With human urine the increase is slight (1–2½ %), with dog's urine as much as 10 %.

7. The presence of dextrose to the extent of 10 % did not affect the estimation of creatine. Phosphoric acid was not found to be superior to hydrochloric in estimating creatine when dextrose is present.

8. The recovery of creatine from diabetic urine gave results lower by 5 % than from aqueous or sugar solutions. Phosphoric acid did not prove more useful for this purpose than hydrochloric. The smaller recovery is probably due to the effect of aceto-acetic acid.

9. The autoclave method gave results identical with those of the water bath. The optimum time for urine and weak solutions of creatine was 25 mins. at 117°–120° C.

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XLVI. NOTE ON THE IODINE CONTENT OF FISH-THYROIDS.

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(*Received Aug. 8th, 1913.*)

The presence of iodine in the tissues of, and oils from, various fishes has been known for a considerable time. It was found in herrings by Jonas [1838], in the oil from the liver of *Raia clavata* by Girardin [1842], and in crabs by Rieger [1853]. Barral [1877] states that iodine is always present in the oils from fish-livers. Stanford [1883] analysed a large number of fish oils, and invariably found iodine present. His analyses seem to have been controlled carefully, although the amounts quoted are minute. Thus:

Cod liver oil (various samples) averaged	0·000322	per cent.	iodine (0·000138—0·000434).
Dry cod fish	contained	0·000829	,,	,,
Scotch herring, salted	,,	0·00065	,,	,,
Scotch herring brine	,,	0·00012	,,	,,
Seal oil	,,	0·00005	,,	,,
Cod liver oil dragées (liver residues from which oil had been removed)	contained	0·05637	,,	,,

Bourcet [1899], using analytical methods similar to those of Baumann, found iodine present in a large number of different species of fish, in amounts somewhat larger than those just quoted, ranging from 0·0007 % (*Leuciscus cephalus*) to 0·024 % (*Merlangus carbonarius*). *Raia clavata* contained 0·002 %. It is uncertain from his published data whether Bourcet's figures refer to fresh or dried fish.

Baumann and Goldmann [1896] fed dried cod to a dog as a probable source of iodine, and confirmed the presence of the element indirectly by noting that such feeding increased the iodine content of the thyroid.

So far as I am aware, fish thyroids have not hitherto been tested for iodine, although in them, as in mammals, its presence is to be expected, and in larger amount than in other tissue. Some indirect evidence on the normal presence of iodine in the thyroids of fishes is afforded by the results of

Marine and Lenhart [1910], who found that the so-called thyroid carcinoma of brook-trout is severe endemic goitre, which can be cured by the administration of iodine as in mammals. This would seem to indicate that these thyroids function in the same way as mammalian thyroids, and hence should contain the same constituents.

While it is still to be proved that iodine is an indispensable constituent of the thyroid, there can be no doubt that it is almost invariably present. Even in those cases where absolutely negative results have been recorded (compare for example Roos [1899], who found that the thyroids of many carnivorous animals contained no trace of iodine), the method employed—that of Baumann and Roos [1895]—is inconclusive for minimal amounts, since it frequently yields too low figures, indicating that minute quantities might entirely escape detection. (See in this connection Seidell [1911].) The actual amount of iodine found varies considerably, and seems to depend largely, if not entirely, on the diet. Roos' results [1899] show that the thyroids of herbivorous animals, whose food contains small quantities of iodine, are richer in iodine than those of carnivorous animals, whose food contains little of the element. For further literature in this connection see Swale Vincent [1912].

There is direct experimental evidence that when iodine compounds are fed the iodine content of the thyroid is markedly increased. Thus Baumann [1896] found that the thyroids of dogs previously fed for some weeks on lean meat (iodine free) contained little or no iodine. That of a dog which had received sheep's thyroids (9 grams) 14 days before death contained 0·34 % (dried gland). Roos [1899] obtained a very similar result. While a large number of analyses showed that the usual iodine content in the dried thyroid of the dog averaged about 0·1 %, that of a dog to which potassium iodide had been administered for some time contained 0·35 %. Baumann and Goldmann [1896] removed the left thyroid of a dog. Its weight, fresh, was 1·92 grams, and the iodine content 0·06 milligram. After the wound had healed, the dog was fed for 14 days on 14 pounds of dried cod (see above). The right thyroid was then removed. It weighed 2·5 grams fresh, 0·8 gram dry, and contained 2·9 milligrams iodine (0·36 %).

It might therefore be expected, taking into consideration the constant presence of iodine in sea-water, that the thyroids of salt-water fishes would contain a maximum amount of iodine. If the conclusions of Gautier [1899] are correct, and the iodine in sea-water is largely present in organised matter, it should be the more capable of assimilation and transmission.

The results given below afford evidence that unusually large amounts are

constantly present in some species, and hence lend support to the view that *the iodine content of the thyroid is a function of the iodine in the diet.*

Through the kindness of the authorities we have been furnished by the Marine Biological Association, Plymouth, with samples of glands of two species of elasmobranchs, *Raia clavata* and *Scyllium canicula.* In all, six samples were received, each consisting of a large number of glands. These had been obtained during the early spring of this year, and stored in absolute alcohol during collection. The alcohol was subsequently evaporated, and the residue added to the glands. The weights of fresh glands given below are therefore only approximate.

The samples were dried in a steam-oven until constant weight was attained, and were analysed by Hunter's method [1910]. This has now been tested by a number of investigators (see for example Seidell [1911]), and is undoubtedly very accurate when appreciable quantities of iodine are present. (In testing for minimal quantities of iodine in other work I have not found this method completely satisfactory, and my results so far seem to support Kendall's conclusions [1912].)

In all, four blank determinations were carried out (one with each series of analyses) using powdered fibrin as organic material; each gave an absolutely negative result. A test of the accuracy of the method, with a known quantity of potassium iodide, gave the following result:

Amount of iodine taken 0·000306 gram.
,, ,, found 0·000310 ,,

This is of a lower order of magnitude than the amounts shown below, and in these the error is probably less. The data for the thyroids are seen in the following table:

Species	Sample	Weight (grams) Fresh	Weight (grams) Dry	Amount taken (gram)	Amount of iodine (gram)	Percentage of iodine (dry substance)
Raia clavata	(1 & 2)	(10·5)	2·541	0·503	0·002208	0·439
				0·503	0·002198	0·437
						(Mean 0·438)
	(3)	(6·4)	1·053	0·513	0·001678	0·327
	(4)	(?)	1·294	0·544	0·001542	0·283
Scyllium canicula	(5)	(4·5)	0·987	0·516	0·003712	0·719
	(6)	(?)	4·393	0·504	0·005831	1·157
				0·524	0·006099	1·164
						(Mean 1·160)

All the thyroids in sample 5 except two were from male specimens and all those in sample 6 from female specimens.

The results may be contrasted with the *maximum* iodine content hitherto observed in different mammals. In all cases the figures indicate percentage iodine in the dried gland.

Maximum iodine content in thyroids.

Fish thyroids	1·160	per cent.		
Dog	0·692	,,	recorded by	Marine and Lenhart [1909, 2].
Human	0·588	,,	,,	,, Seidell [1911].
Stag	0·54	,,	,,	,, Blum [1899].
Pig	0·531	,,	,,	,, Seidell and Fenger [1913] (fat-free).
Sheep	0·53	,,	,,	,, Baumann and Roos [1895, 1].
Beef	0·477	,,	,,	,, Marine and Lenhart [1909, 1].
Goat	0·28	,,	,,	,, Blum [1899].

The *average* iodine content can probably be taken as from 30 to 50 % of these figures.

While the thyroids of *Raia clavata* only contain a quantity of iodine of the same order as these maxima, both samples of *Scyllium canicula* contain more, and one *much more iodine than any thyroid previously reported on.* This is the more remarkable since this figure is the average for a large number of glands, while several of the other maxima (including the next three highest figures) are for single individuals. The theory that the iodine content of the diet plays a considerable, if not the whole, rôle in determining that of the gland therefore receives strong support from these figures.

The different results from the two sexes in *Scyllium* are possibly accidental. No previous marked variation seems to have been noted.

I hope to examine other species shortly, and to contrast thyroid with other tissue in what seems most probably the optimum condition for maximal iodine content.

I wish to thank Professor Swale Vincent for his kind interest in this work.

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XLVII. THE COMBINATIONS OF HAEMOGLOBIN WITH OXYGEN AND WITH CARBON MONOXIDE. I.

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(*Received August 16th, 1913.*)

In a previous paper Barcroft and Hill [1910] gave evidence to show that in dialysed solution haemoglobin has its smallest possible molecular weight, contains, namely, one atom of iron. In a later communication Hill [1910] suggested that the dissociation curves of oxyhaemoglobin in the presence of salts and carbon dioxide can be calculated from the hypothesis that these bodies tend to aggregate the large haemoglobin molecules into larger molecules, which then combine with oxygen according to the equation

$$Hb_n + nO_2 \rightleftarrows Hb_n(O_2)_n.$$

If y be the percentage saturation of the haemoglobin with O_2, and x the tension of the latter in the solution, it can be shown that this hypothesis leads to an equation for the dissociation curve of the type

$$y = 100\frac{Kx^n}{1+Kx^n} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1),$$

where K is the equilibrium constant and n is a whole number > 1.

This equation seems to suit all known dissociation curves of oxyhaemoglobin with a very high degree of accuracy, as numerous published and unpublished experiments of Barcroft [1913] and others (see e.g. Douglas, Haldane, J. S. and Haldane, J. B. S. [1912]) will show. In point of fact n does not turn out to be a whole number, but this is due simply to the fact that aggregation is not into one particular type of molecule, but rather into a whole series of different molecules: so that equation (1) is a rough mathematical expression for the sum of several similar quantities with n equal to 1, 2, 3, 4 and possibly higher integers.

The basis of the suggestion lies fundamentally in the idea that the

oxygenated haemoglobin molecules consist almost entirely of the fully saturated types

$$HbO_2,\ Hb_2(O_2)_2,\ Hb_3(O_2)_3,\ \text{etc.},$$

rather than of the partially saturated types Hb_2O_2, Hb_3O_2, Hb_4O_2, etc. For the observed dissociation curves in the presence of salts exhibit a double curvature (i.e. are of an **S**-shape), differing therein from the dissociation curve of dialysed haemoglobin, which latter is a rectangular hyperbola always concave to the horizontal axis. The former dissociation curves start from the origin almost—if not quite—horizontally, bend upward at first with concavity facing the y-axis, pass through a point of inflexion, and then bend inward again with concavity towards the x-axis. This main property of the curves is simply represented physically by the idea that the aggregated haemoglobin molecule, taken e.g. to be Hb_2, is oxidised into the form $Hb_2(O_2)_2$ by combination with two oxygen molecules simultaneously, and that the unsaturated molecule Hb_2O_2 either does not exist at all or exists in negligibly small quantities. For in the former case the dissociation curve is of the form $y = 100\frac{Kx^2}{1+Kx^2}$, the tangent to which at the origin is $y = 0$, i.e. the horizontal axis: and this approximates to the fact observed experimentally. While if the unsaturated Hb_2O_2 exists in appreciable quantities the dissociation curve involves an equation of the type $y = B\frac{Kx}{1+Kx} + \text{etc.}$, where B is some constant less than 100, the tangent to which curve at the origin is $y = BKx$, a line going out at a slope as in the dissociation curve of dialysed haemoglobin, and not as observed when salts are present.

Now it seems at first sight almost unreasonable to suppose that Hb_2 would combine only with $2O_2$, i.e. with two molecules at once, and would not oxidise partially at first by combination with the single molecule O_2: that in fact the oxidation goes on according to the equation

$$Hb_2 + 2O_2 \rightleftarrows Hb_2(O_2)_2$$

and not according to the equations

$$\left.\begin{aligned} Hb_2 + O_2 &\rightleftarrows Hb_2O_2 \\ \text{and then}\quad Hb_2O_2 + O_2 &\rightleftarrows Hb_2(O_2)_2 \end{aligned}\right\} \quad \text{(A)}.$$

One could see no analogy for an equation of the former type when the latter scheme was so obviously possible. That the latter scheme does not immediately satisfy the facts is obvious from a short calculation, which however I believe will lead us to the clue to the whole matter. In equations (A) above, if K and K' be the equilibrium constants of the two reactions respectively, and

if the concentration of Hb_2O_2 be u, of $Hb_2(O_2)_2$ be v, of Hb_2 be w, and if x be the tension of oxygen and y the % saturation we find from the laws of mass-action

$$\frac{u}{wx} = K \text{ and } \frac{v}{ux} = K'.$$

Also since $(u+2v)$ molecules of Hb are saturated with O_2, and there are $(2u+2v+2w)$ molecules of Hb altogether,

$$y = 100\,\frac{u+2v}{2u+2v+2w},$$

which becomes

$$y = 100\,\frac{Kx+2K'Kx^2}{2Kx+2K'Kx^2+2},$$

when we put $u = Kwx$ and $v = K'xu = KK'x^2w$.

The tangent at the origin to this curve is

$$2y = 100Kx,$$

a straight line which is not the horizontal axis; thus the scheme represented by equations (A) does not give a dissociation curve of the observed kind—coming in horizontally to the origin—*unless K is exceedingly small.*

If however K is exceedingly small the difficulty entirely disappears, and the equation becomes

$$2y = 100\,\frac{KK'x^2}{KK'x^2+1},$$

which is of the type required to fit the dissociation curve, with tangent at the origin $y = 0$. (It should be noted that if K is small K' must be large, so that KK' is finite.)

Can we therefore assume that K is small, that in fact oxygen combines far more readily with Hb_2O_2 than it does with Hb_2? In other words, is it a justifiable assumption that the partially saturated body Hb_2O_2 is very unstable, and exists in almost inappreciable quantities: that in fact Hb_2O_2 once formed almost immediately combines with another O_2 to form the saturated compound $Hb_2(O_2)_2$? In support of this contention[1] one may argue that there is no transitional spectrum corresponding to unsaturated oxides, and therefore that, if there is any truth in the aggregation theory, the unsaturated oxides are present only in negligible quantities. The theory will obviously lead directly to a dissociation curve of the observed type, to which by adjusting the value of KK' it can be made to conform with some degree of accuracy. So far then as we are concerned with dissociation curves of oxyhaemoglobin, the equations to these can be deduced at once from the

[1] First made to Mr Barcroft by Mr W. H. Mills of the Chemical Laboratory, Cambridge.

aggregation hypothesis with the conception of gradual combination with oxygen according to the scheme

$$Hb_n + O_2 \rightleftarrows Hb_nO_2,$$
$$Hb_nO_2 + O_2 \rightleftarrows Hb_n(O_2)_2,$$
$$Hb_n(O_2)_2 + O_2 \rightleftarrows Hb_n(O_2)_3, \text{ etc.},$$

provided always that we may assume that O_2 combines much more readily with Hb_nO_2 to form $Hb_n(O_2)_2$, than with Hb_n to form Hb_nO_2: *in other words that the partially saturated molecule is very unstable, is difficult to form and easy to combine further with oxygen.* For all such combinations lead to a dissociation curve with double curvature, which reaches the origin horizontally.

A plausible suggestion as to the reason why O_2 should combine much more readily with Hb_2O_2 than with Hb_2 may be put forward here. The haemoglobin molecules are probably aggregated by surface effects, by the neutralisation e.g. of the electric charges on their surfaces. It may be however that the free chemical "bonds" of Hb, with which reduced haemoglobin attaches itself to oxygen, are in addition combined with one another in the aggregated molecule, so that if reduced haemoglobin is Hb= then the aggregated molecule is not merely Hb, Hb (two molecules just sticking together by surface forces) but rather Hb=Hb. In that case Hb_2O_2, the half saturated molecule, has to be formed by the breaking loose of these bonds, and will be =Hb, Hb=O_2. This body one would naturally expect to be very unstable, as is Hb= in the presence of oxygen. It would be very difficult for O_2 to form this compound, because it has to break down the Hb=Hb molecule into =Hb, Hb= in order to do it: and the compound =Hb, Hb=O_2 once formed, presenting as it does two unsaturated bonds, would immediately seize on another O_2 molecule becoming O_2=Hb, Hb=O_2. Whether this turns out to be the case or not, it presents a very clear physical conception as to why Hb_2O_2 should exist in only very minute concentrations.

So far then the aggregation hypothesis can be made to fit the observed facts very closely, and to have a reasonable physical explanation. Some doubt has been thrown on the particular form of it advanced in my earlier paper, by Douglas, Haldane and Haldane [1912]. These authors determined experimentally the CO-haemoglobin curve in the presence of a partial pressure of oxygen, and found it to be a rectangular hyperbola. According to the rough scheme, dealing with single molecules,

$$HbO_2 + CO \rightleftarrows HbCO + O_2,$$

(and supposing the haemoglobin to be all combined with either CO or O_2) the equation to the CO-dissociation curve in the presence of a constant partial pressure of O_2 should be that of a rectangular hyperbola. But dealing with aggregated molecules, say Hb_2, the formula

$$Hb_2(O_2)_2 + 2CO \rightleftarrows Hb_2(CO)_2 + 2O_2$$

leads, by the laws of mass action, to an equation $\frac{y'x^2}{yx'^2} = K$, if x and x' be the

partial pressures of O_2 and CO, and y and y' be the % saturations with O_2 and CO. If all the haemoglobin is combined $y + y' = 100$, so that

$$\frac{y'}{100 - y'} = K \frac{x'^2}{x^2},$$

which represents a CO-dissociation curve of the type described above, containing a point of inflexion, and coming in horizontally to the origin. Thus this, and as a matter of fact all similar conceptions, of the balanced action between CO, O_2 and haemoglobin, led to the same type of equation, which gives a curve in no way like a rectangular hyperbola: so that the experiment of Douglas and Haldane led one to doubt the validity of the whole hypothesis of aggregation, in spite of the accuracy with which it fitted some of the facts. *Why is it that the CO-O_2-haemoglobin dissociation curves, in the presence of a constant tension of O_2, are unaffected by salts and CO_2, when the O_2-dissociation curves and the CO-dissociation curves are so very largely affected?* This very puzzling question can, as a matter of fact, be quite simply answered by the aid of the conception sketched above.

Let us assume for simplicity that the haemoglobin exists as double Hb_2 molecules, and that according to the following scheme it combines gradually and by steps with CO and O_2. The Hb_2 combines first with one O_2 or with one CO, the new molecule Hb_2O_2 or Hb_2CO combines further with O_2, or CO, to form one of the three compounds

$$Hb_2(O_2)_2, \quad Hb_2(O_2)(CO), \quad \text{or} \quad Hb_2(CO)_2.$$

We then have the equations (in which the small letters represent concentrations and the k's represent equilibrium constants)

$$\left.\begin{array}{lll}
\underset{y}{Hb_2} + \underset{x}{O_2} \rightleftarrows \underset{u}{Hb_2O_2} & k_1 \\
\underset{u}{Hb_2O_2} + \underset{x}{O_2} \rightleftarrows \underset{v}{Hb_2(O_2)_2} & k_2 \\
\underset{u}{Hb_2O_2} + \underset{x'}{CO} \rightleftarrows \underset{w}{Hb_2(O_2)(CO)} & k_3 \\
\underset{y}{Hb_2} + \underset{x'}{CO} \rightleftarrows \underset{u'}{Hb_2CO} & k_1' \\
\underset{u'}{Hb_2CO} + \underset{x'}{CO} \rightleftarrows \underset{v'}{Hb_2(CO)_2} & k_2' \\
\underset{u'}{Hb_2CO} + \underset{x}{O_2} \rightleftarrows \underset{w}{Hb_2(CO)(O_2)} & k_3'
\end{array}\right\} \quad \text{.....(A).}$$

From these, by the laws of mass action, we find

$$\text{I.} \quad \frac{u}{xy} = k_1, \quad \frac{u'}{x'y} = k_1',$$

$$\text{II.} \quad \frac{v}{ux} = k_2, \quad \frac{v'}{u'x'} = k_2',$$

$$\text{III.} \quad \frac{w}{ux'} = k_3, \quad \frac{w}{u'x} = k_3'.$$

From equations I and II, multiplying up, we find

$$\text{IV.} \qquad \frac{v}{x^2y} = k_1k_2, \qquad \frac{v'}{x'^2y} = k_1'k_2',$$

and from equations I and III

$$\text{V.} \qquad \frac{w}{xx'y} = k_1k_3, \qquad \frac{w}{xx'y} = k_1'k_3',$$

so that $k_1k_2 = k_1'k_3' = k$ say.

Let ξ be the amount of haemoglobin per c.c. saturated with O_2: ξ' the amount saturated with CO. Then

$$\xi = \tfrac{1}{2}(u+w)+v,$$
$$\xi' = \tfrac{1}{2}(u'+w)+v'.$$

Using the values of u, v and w given in equations I, IV and V we find

$$\left.\begin{aligned} \xi &= \tfrac{1}{2}(k_1xy + kxx'y) + k_1k_2x^2y \\ \text{and} \quad \xi' &= \tfrac{1}{2}(k_1'x'y + kxx'y) + k_1'k_2'x'^2y \end{aligned}\right\} \text{.....(B).}$$

$$\therefore \quad \frac{\xi'}{\xi} = \frac{x'}{x} \times \frac{\tfrac{1}{2}(k_1'+kx)+k_1'k_2'x'}{\tfrac{1}{2}(k_1+kx')+k_1k_2x}.$$

Thus apparently the % saturations of the Hb with O_2 and CO are *not* in the direct ratio of the latter's tensions, as is necessary if the dissociation curve is to be a rectangular hyperbola. We come however to the hypothesis outlined above, viz. that the unsaturated Hb_2O_2 is very unstable and tends to break down either into Hb_2 or Hb_2O_4. In this case we may assume that k_1 is very small, and k_2 very large, their product remaining finite. Similarly we have assumed that k_1' is very small and k_2' is very large. We therefore find, neglecting k_1' and k_1,

$$\frac{\xi'}{\xi} = \frac{x'}{x}\,\frac{\tfrac{1}{2}kx + k_1'k_2'x'}{\tfrac{1}{2}kx' + k_1k_2x} \qquad \text{.....(C).}$$

This, again, is not a direct proportion between ξ'/ξ and x'/x, as we should expect from Douglas and Haldane's observations [1912, see especially pp. 278, 290]. Looking however at the chemical equations (A) above, if we were to assume that CO has a very much higher affinity for the partially saturated $= Hb, Hb = O_2$ (i.e. Hb_2O_2) than has O_2, i.e. that nearly all the Hb_2O_2 combines with CO rather than with O_2: and if we further assume that the partially saturated $= Hb, Hb = CO$ (i.e. Hb_2CO) has a much higher affinity for the CO than it has for the O_2, i.e. that nearly all the Hb_2CO combines with CO rather than with O_2, then the whole difficulty is immediately solved. For[1] in the first case we find that k_2x' is very much greater than k_2x, and

[1] It should be noted that the assumption is not simply that Hb_2O_2 has a greater affinity for a given pressure of CO than it has for the same pressure of O_2: this, of course, we should expect. It is that the affinity of Hb_2O_2 for CO exceeds the affinity of Hb_2O_2 for O_2 more than the affinity of Hb for CO exceeds the affinity of Hb for O_2: that the CO-affinity is even greater, relatively to the O_2-affinity, in the aggregated molecule than it is in the simple.

in the second that $k_2'x'$ is much greater than $k_3'x$. These assumptions are in the highest degree reasonable, and making them, equation (C) above immediately gives the required result. Putting $k = k_1'k_2'$ in the numerator and $k = k_1k_3$ in the denominator (as shown above) we find

$$\frac{\xi'}{\xi} = \frac{x'}{x}\frac{k_1'}{k_1}\frac{\frac{1}{2}k_3'x + k_2'x'}{\frac{1}{2}k_3x' + k_2x}$$

and assuming, as above, that $k_3'x$ can be neglected in comparison with $k_2'x'$, and that k_2x can be neglected in comparison with k_3x', we find

$$\frac{\xi'}{\xi} = \frac{x'}{x}\frac{k_1'}{k_1}\frac{k_2'x'}{\frac{1}{2}k_3x'}$$
$$= \frac{x'}{x}\frac{2k_1'k_2'}{k_1k_3}.$$

That is to say, the CO- and O_2-saturations of the haemoglobin are proportional to their partial pressures, and if the haemoglobin be fully saturated with CO and O_2, i.e. putting $\xi + \xi'$ equal to a constant (say 100) we find, if $2k_1'k_2'/(k_1k_3) = K$,

$$\frac{\xi'}{100 - \xi'} = \frac{x'}{x} \times K.$$

This is a rectangular hyperbola between ξ', the % saturation with CO, and x', the partial pressure of the CO. Thus on quite simple assumptions the theory is found to agree perfectly with the experimental observations of Douglas and Haldane.

Now Douglas and Haldane have brought to light some other experimental facts which seem at first sight to be difficult of explanation. They found [1912, p. 279] that the CO-O_2-haemoglobin dissociation curve, in the presence of a fixed tension of CO and a variable tension of O_2, was a rectangular hyperbola for large tensions of oxygen but bent back and fell again as the O_2-tension became very small and gradually vanished. Now, on our theory as advanced above, a very pretty explanation of this fact can be found, which may elicit some facts of further physiological interest. The assumption made above is practically that CO combines much more readily with Hb_2O_2 in the presence of O_2, than it does with Hb_2 in the presence of the same O_2. When the O_2-pressure is reduced beyond a certain limit very little Hb_2O_2 is formed, and therefore the CO is forced to combine with Hb_2 direct—which it does much less readily, and therefore the curve falls again. An analytical expression of this fact is given below. In order to find the dissociation curve in question we have to find the relation between ξ', the CO %-saturation, and x, the O_2-tension. We know that the total amount of haemoglobin present can be represented by 100, so that, neglecting the small amounts of the unsaturated compounds present,

$$2\xi + 2\xi' + 2y = 100.$$

Putting into this equation the values of ξ and ξ' in terms of y given in equations (B) above, we find

$$2y = \frac{100}{1+\frac{1}{2}(k_1x+k_1'x')+kxx'+k_1k_2x^2+k_1'k_2'x'^2},$$

so that, from equation (B)

$$\xi' = \frac{100\,(k_1'k_2'x'^2+\frac{1}{2}k_1'x'+\frac{1}{2}kxx')}{1+\frac{1}{2}(k_1x+k_1'x')+kxx'+k_1k_2x^2+k_1'k_2'x'^2}.$$

This relation between ξ', the $^0/_0$-saturation with CO, and x, the O_2-tension, is too complicated for simple calculation and comparison with experimental results. It can however be shown that it may possess the general qualities of the experimental curve found by Douglas and Haldane [1912, p. 279]. It rises from the horizontal axis in much the same way as their curve, with diminishing O_2-tension, and then reaches a maximum and falls again slightly as the O_2-tension becomes very small and finally vanishes. This can be simply tested by finding the value of $\partial\xi'/\partial x$ at $x=0$, i.e. the slope of the curve at the point of zero O_2-pressure. If $\partial\xi'/\partial x$ is positive, i.e. if the curve slopes upwards at first, ξ' first increases and then of course later decreases as x increases. If $\partial\xi'/\partial x$ is negative at $x=0$, then the curve slopes down from the beginning.

We should expect therefore, from Douglas and Haldane's results, that the value of $\partial\xi'/\partial x$ at $x=0$ should be >0.

A simple calculation will show that

$$\frac{1}{100}\left(\frac{\partial\xi'}{\partial x}\right)_{x=0} = \frac{\frac{1}{2}kx'+\frac{1}{2}kk_1'x'^2+kk_1'k_2'x'^3-k_1k_1'k_2'x'^2-\frac{1}{2}k_1k_1'x'-2kk_1'k_2'x'^3-kk_1'x'^2}{(1+k_1'x'+2k_1'k_2'x'^2)^2}.$$

Let us assume that x' is very large, i.e. that there is a large tension of CO. Then $(\partial\xi'/\partial x)_{x=0}$ is obviously negative, because of the preponderating negative value of the term $-2kk_1'k_2'x'^3$. Hence Douglas and Haldane's curve will *not* exhibit a fall of CO $^0/_0$-saturation as the O_2-tension is diminished to zero, if the CO-tension is high. The assistance provided to the combination of CO by the presence of a little unsaturated Hb_2O_2 is of no importance if the CO-tension is very high. Let us assume that x' is very small. Then the value of $\frac{1}{100}(\partial\xi'/\partial x)_{x=0}$ is

$$\frac{\frac{1}{2}kx'-\frac{1}{2}k_1k_1'x'}{(1+k_1'x')^2},$$

neglecting squares or higher powers of x'. This quantity is therefore positive if $k>k_1k_1'$. Now $k=k_1k_2$. Hence we must have $k_2>k_1'$. This we should expect to be the case. From the assumption already made, k_2' is very much greater than k_1', CO combines much more readily with the partially saturated Hb_2CO than with the completely unsaturated Hb_2: one

would expect it therefore to combine much more readily with the partially saturated Hb_2O_2 than with the completely unsaturated Hb_2. In that case k_2 would be as much greater than k_1', as k_2' is: and on the latter inequality the whole of the argument of this paper is based. Without therefore introducing any new hypothesis we have shown that, for a small constant CO-tension, the CO-O_2-dissociation curve in the presence of an increasing tension of O_2 rises at first, reaches a maximum and falls slowly to zero, as in Douglas and Haldane's recorded observations.

In all the above considerations Hb_2 has been taken as the general type of the molecular cluster. Any other type of molecule, Hb_3, Hb_4, ..., can be considered of course in exactly the same way: the mathematical treatment would be more complicated, although exactly the same in type.

Douglas, Haldane and Haldane in their paper [1912, p. 296] put forward an ingenious theory to account for the several dissociation curves observed, both with O_2 and CO: and they gave an equation for a dissociation curve which fits the experimental points fairly closely, but not so closely as does the equation deduced from the hypothesis of this paper. In order to arrive at this equation they made various assumptions as to the exact values of the dissociation constants of the various reactions, for which, as they admit [1912, p. 301], there are no real *a priori* reasons: and there are other improbabilities in their assumptions, at least as great as in those made above in this paper. For example the idea that "the aggregated molecules do not give up or take up O_2 or CO without first splitting up into simple molecules" seems difficult to admit: and it is not easy to understand, if oxyhaemoglobin molecules are continually aggregating and reduced molecules are continually aggregating, how the oxyhaemoglobin and the reduced haemoglobin molecules do not aggregate together to form unsaturated clumps. It is true however that their theory does agree to a considerable extent with observed facts, even though they have made many rather difficult assumptions in the development of it: so that, even though the theory sketched in this paper seems to me to be more probable, I do not feel that one can as yet decide definitely between the two. It is noticeable however (as Barcroft shows) that the equation to the dissociation curve given here, and in the previous paper, does definitely suit the experimental facts better than the equation deduced from the hypothesis of Douglas, Haldane and Haldane. That a modification however of their assumptions would make their equation fit the experimental facts better than at present, seems to me very probable: so that a judgment between their theory and mine can scarcely be given on the facts hitherto discussed. At present however the theory and assumptions

given here seem to me to be simpler and easier, as well as to fit the experimental data better, than the hypothesis advanced by Haldane, Douglas and Haldane.

Summary and Conclusions.

The O_2- and CO-dissociation curves of haemoglobin are known to differ, according as salts and CO_2 are present or are not. A theory to explain this has already been advanced, viz. that the simple molecules of haemoglobin are aggregated into molecular clusters. This theory explained many of the facts very exactly, but certain objections had been raised to it in particular by Douglas, Haldane and Haldane, especially in relation to the CO-O_2-dissociation curves.

It is shown here that the theory is capable of including all the known facts in relation to CO- and O_2-dissociation curves, if one is allowed to make certain simple assumptions as to the order of magnitude of the equilibrium constants in the several reactions involved. These are:

(i) that the half saturated molecules Hb_2O_2 and Hb_2CO are very unstable, and change at once into either Hb_2 or $Hb_2(O_2)_2$, $Hb_2(CO)_2$ or $Hb_2(CO)(O_2)$,

(ii) that the half saturated molecules Hb_2O_2 and Hb_2CO combine much more readily with CO than with O_2.

The first of these assumptions can be explained as due to the fact that Hb_2 is $Hb = Hb$, while Hb_2O_2 is $= Hb, Hb = O_2$, with two unsaturated bonds, which tends to combine at once with more O_2 to form $O_2 = Hb, Hb = O_2$.

If these assumptions are justified, we may deduce that since CO combines much more readily with Hb_2O_2 than with Hb_2, haemoglobin will take up more CO at a given tension, if a little O_2 is present than if O_2 is completely absent.

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XLVIII. THE COMBINATIONS OF HAEMOGLOBIN WITH OXYGEN AND WITH CARBON MONOXIDE. II.

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(*Received Aug. 16th, 1913.*)

In the preceding paper Hill [1913] has expanded his formula

$$y/100 = \frac{Kx^n}{1+Kx^n}$$

in which $y =$ the percentage saturation of the haemoglobin with oxygen, x the oxygen pressure, K the equilibrium constant of the reaction and n the average number of molecules of haemoglobin in each aggregate. The formula was originally put forward [Hill, 1910] to cover the dissociation curves of haemoglobin in pure aqueous solution and in certain saline solutions. With the introduction of certain assumptions it may now be applied (1) to the affinity of blood for oxygen in the absence of carbonic acid, (2) to the affinity of blood for carbon monoxide in the absence of oxygen, (3) the partition of haemoglobin between oxygen and carbon monoxide in the presence of the two gases and either with or without acids such as CO_2 in the system. The validity of the theory depends not only upon the soundness of the reasoning on which it rests, but upon the accuracy with which it fits the vast number of experimental data by which it may be tested.

It may here be pointed out that the formula contains but two constants n and k: the experimental test therefore is a much more crucial one than in the case of the more adaptable formula of Douglas, Haldane and Haldane [1912], which contains three constants.

Since the formula was first published by Hill, I have devoted a great deal of attention to the accuracy with which it fits the facts. The correspondence is so striking that I propose to record it in the present paper.

I will treat of the evidence as regards

(1) the reaction $Hb + O_2 \rightleftarrows HbO_2$,

where the haemoglobin is dissolved in solutions of various salts,

(2) $Hb + O_2 \rightleftarrows HbO_2$ with reference to blood.

Haemoglobin in Saline Solutions.

In Hill's preliminary communication he tabulated the comparison of the curves calculated from the formula and the freehand curves drawn by Camis and myself [1910] for solutions of haemoglobin in water, 0·7 % NaCl and 0·9 % KCl.

The figures lost much of their force from want of a fuller presentation. It was not evident from a casual study of these data that all the curves they represented for which the value of n was more than unity were in essence **S**-shaped. It therefore seemed that there was an essential difference between these curves and those which were known to exist for blood, the latter being **S**-shaped curves.

Blood.

Blood offers a far more searching test than haemoglobin solution because the curves are much more spread out. There is no case on record in which a great number of determinations, say more than about a dozen, have been carried out on the blood of any one person at one time. Fortunately the blood of the persons who have been most thoroughly investigated seems the same at one time as at another[1]; a single curve may be taken as representing the blood of one individual, for instance Douglas, Zuntz or myself, irrespective of the time at which the determinations were made.

Thirty-eight determinations of points on Douglas' normal dissociation curve at his existing alveolar CO_2 pressure of 40–41 mm. have been made. The series is especially valuable because the determinations have been made by somewhat different methods, by different persons, and on different scales. Rather more than half were made by me, and for all these the differential method of blood gas analysis was used, dilute ammonia unaided by saponin being employed in the blood gas bottles and in most cases 0·1 c.c. of blood being used for each analysis. Sixteen of the points shown were determined by Haldane and Douglas; they did not use the differential apparatus, but Brodie's adaptation of the old Barcroft-Haldane apparatus. They used 1 c.c. of blood for their analyses. Five determinations were made with dilute sodium carbonate as the fluid for taking the blood whilst the rest were made with ammonia. They used saponin in addition to the alkali. Moreover, Haldane and Douglas treated the gas in their tonometer somewhat differently from the way in which I did. Their method involved them in much greater corrections for the changes during analysis in gases held physically in solution. In spite of this their method was probably somewhat more accurate than mine.

[1] Barcroft [1911], Douglas, Haldane and Haldane [1912, p. 283].

In treating of the data I have adopted two methods. The first is to take all the data in order, to divide them into three groups as nearly as possible equal, to state (1) the difference between the observed and the calculated percentage saturation in the case of each observation, (2) the greatest difference in each direction and the mean difference in the case of each group and (3) the same in the case of the whole series. The following is the result:

Pressure obs.	% sat. obs.	Dev. from curve	Pressure obs.	% sat. obs.	Dev. from curve	Pressure obs.	% sat. obs.	Dev. from curve
9	6	+1	36	63	+0·5	59	84	−1
11	6	+3	36	67	+4·5	61	83	−3
17·5	26	+3	37	63	−1	61	84	−2
18	24	+1	38	65	−1	70	89	−0·5
20	27	−0·5	39	71	+3	77	92	+0·5
20	30	+2·5	39	67	−1	85	92	−1·5
23	38	+3	41	71	+1	85	94	+0·5
24	37	−1	42	75	+4	90	92	−2·5
26	39	−3	42	72	+1	95	99	+4
29	51	+2	49	77	−1·5	97	94	−1·5
30	50	−1	49	79	+0·5	96	96	+0·5
34	57	−2	53	78	−3	104	96	0
			59	81·5	−3·5	104	97	+1

	Greatest error	Mean
Total of 38	+4·5 −3·5	0·14 %

The second plan of treating the results is to divide according to the methods by which they were obtained. Taking the 16 results obtained by Haldane and Douglas, eight are above the line and eight below it. The greatest individual discrepancies are + 4 % and − 3 %. The mean of the sixteen is 0·19 %. Of their sixteen results one differs from the calculated number by 4 %, three by 3 %, three by 1·5–2 %, eight by 0·5–1 % and one by under 0·5 %: whilst of the whole series of thirty-eight, there is one of 4·5 %, three of 3·5–4 %, nine of 2·5–3 %, six of 1·5–2 %, eighteen of 0·5–1 % and one of under 0·5 %. The general run of the figures is so nearly the same in each of the two series, as to prove that the method of experiment does not affect the comparison at issue.

Nine points have been determined on the Zuntz blood at 40 mm. CO_2 pressure. I could not find that it differed from that of Douglas, it is therefore referable to the same curve. Of these nine, three fall on the curve (within 0·5 % of it), four are below it and two are above it.

Oxygen pressure	0	12	30	37	37	43	61	79	97
Observed % saturation	0	4	50	61	61·5	72	86·5	92	95
Divergence from curve	0	−5	−1	−4	−3·5	0	+·5	+1	0

Extreme divergence $\begin{cases} +1 \\ -5 \end{cases}$. Mean divergence −1·3 %.

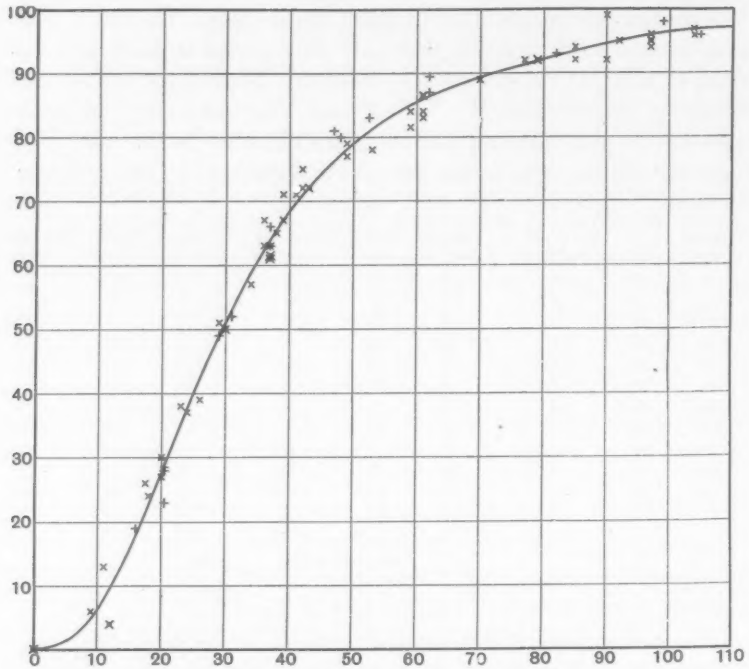

Fig. 1. Ordinate = percentage saturation with oxygen; abscissa = oxygen pressure.
× Blood of Douglas. ⧓ Blood of Zuntz. + Blood of Haldane.

Referable to this curve also is the blood of Haldane which I have taken from the figure in the *Journal of Physiology*, vol. XLIV, page 283, as faithfully as the scale of the figure permits. The points are as follows:

Oxygen pressure	26	20·3	20·5	20·5	29	31	37	37	47	48	52·5	62	62	82	99	105
Observed % sat.	19	28·5	28	23	49	52	63	66	81	80	83	87	89·5	93	98	96
Div. from curve	0	−0·5	0	−5	0	+0·5	−1	+2	+4	+2	+1	0	+2·5	+1	+2·5	0

Extreme divergence $\begin{cases} +4 \\ -5 \end{cases}$. Mean divergence 0·6 %.

The results of the comparisons between the curve drawn from the equation, and the actual determinations which I have cited may be summarised as follows. (1) Of the 63 determinations none differs by more than 5 % from the calculated value, (2) the "average error" reckoning all departures from the calculated value as positive quantities is 1·6 %, (3) the mean of all the errors reckoning those above the line as positive and those below it as negative is 0·02 %.

It is known that the blood of different persons differs. I have compared the points which have been determined on my own blood with the curve, the constants of which are $n = 2{\cdot}5$, $K = 0{\cdot}000292$. Only about 20 points are available for the comparison.

Dividing them into two groups they are as follows:

Pressure of oxygen	0	1·5	7	13	22	28	36	36	37	38·5
% sat. observed	−1	2	4	12	37	55	69·5	71	68	72·5
Div. from calcd. value	−1	+1·5	0	−4	−2	0	0	+1·5	−2·5	+0·5

Extreme divergence $\begin{cases} +1{\cdot}5 \\ -4 \end{cases}$. Mean −0·6 %.

Pressure of oxygen	38·5	43	50	50	50	50	57	65	65	65	89
% sat. observed	74·5	79·5	83·5	83·5	84·5	85	89	89	90	90·5	95
Div. from calcd. value	+2·5	+1	−0·5	−0·5	+0·5	+1	0	−3	−2	−1·5	0

Extreme divergence $\begin{cases} +2{\cdot}5 \\ -3 \end{cases}$. Mean −0·23 %.

Taking the two series together the results may be summarised as follows: (1) no single determination differs by more than 4 % from the calculated value, (2) the "average error," calculating all errors as positive, is 1·2 %, (3) the mean divergence, calculating points above the line as positive and those below as negative, is 0·36 %.

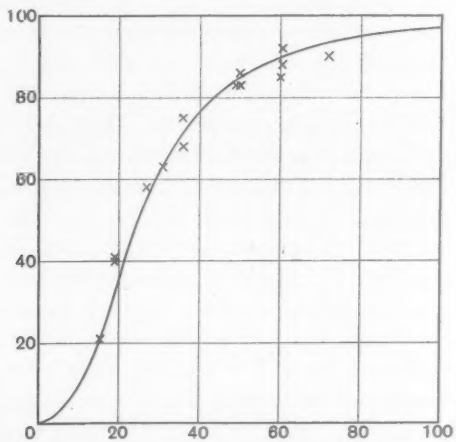

Fig. 2. Zuntz[1] dissociation curve, 35 mm. CO_2; ordinate = percentage saturation with oxygen, abscissa = oxygen pressure in mm.

Of the cases of human blood which have been studied at all exhaustively by me, the least satisfactory from the present point of view is that of Zuntz at his existing alveolar CO_2 pressure of 34–35 mm. of CO_2; here, however,

[1] The data and pressure given on p. 62, *Journal of Physiol.* Vol. XLII, are slightly erroneous owing to a wrong correction for the zero of the gas burette.

the chief cause of discrepancy is a point done in duplicate (pressure 19 mm. percentage saturation 41 and 40 %). The data are as follows. The curve here is very steep, and a slight error in the pressure measurement would account for it.

Pressure of oxygen	15·5	19	19	23	27	31	36	36	49	50	50	60	60·5	60·5	72
% sat. observed	21	40	40	50	58	63	75	68	83	86	83	85	92	88	90
Div. from calcd. value	−1	+6	+7	+4	+3	0	+4	−3	−1	+1	−2	−5	+2	−2	−3

Of these fifteen points one is on the line, seven above and seven below it, the average error counting all errors as positive is 3 %, whilst the mean counting errors above the line, as positive, and those below it, as negative, is 0·66 %.

So far as I know there are no other cases in which a sufficient number of points has been determined and these spread sufficiently uniformly over the curve to test the truth or otherwise of the theory.

At this point a few words may be said by way of comparison between the curve obtained from Hill's formula and that obtained from the formula of Douglas, Haldane and Haldane. Over a great portion of the curves obtained from the two formulae they agree so closely as to make an experimental test between the one and the other almost impossible.

At pressures below 20 mm. the two curves differ and it may be well to compare this portion of them with the observed points. This task is made easier because at very low oxygen pressures the difference between the blood of different persons is very slight and therefore the determinations—all too few—for human blood may be taken together for the purpose. This may be done the more freely by me because such error as there is in the application of results obtained from my blood to the curves of Douglas' blood which are those depicted, would favour the formula of Douglas, Haldane and Haldane.

Below 15 mm. there appear to be eleven determinations on human blood, of these the three lowest, at 0 and 1·5 mm. offer no test of the relative merits of the two curves, yet I have included them because they are all important to the argument as showing that the percentage saturations as observed start from the zero. Had there been some such error as incomplete laking of the blood or wrong correction for the gases in physical solution these points would not have fallen on the base line.

Below are the data of the eleven points.

Pressure of oxygen	0	0	1·5	6	7	8	9	11	12	12	13
Percentage saturation observed	0	−1	2	−1	4	+1	6	13	4	10	12
Divergence from Haldane's curve	0	−1	+1	−6	−3	−7	−3·5	+1	−10	−4	−3·5
Divergence from Hill's curve ...	0	−1	+2	−2·5	+1	−2·5	+1·5	+5·5	−5	+1	+1

Averaging the points between 5 and 15 mm., (1) the extreme departures from Haldane's formula are $+1$ and -10 and from Hill's $+5{\cdot}5$ and -5, (2) reckoning the "average error" (counting all the errors as positive) it is with regard to Haldane's formula $4{\cdot}8\,\%$ and with regard to Hill's $2{\cdot}5\,\%$, (3) reckoning errors above the line as positive and below it as negative, the mean divergence from Haldane's curve would be $4{\cdot}5\,\%$ and from Hill's curve $1{\cdot}2\,\%$. It seems clear from the figures and more clear from Fig. 3, that Hill's formula more closely represents the facts than does that of Haldane. Eight points is however a small number to take for such a purpose and while it is evident that the equation $y/100 = \frac{Kx^n}{1+Kx^n}$ has the better of the comparison, many more points would have to be determined before the argument could be regarded as settled on this count.

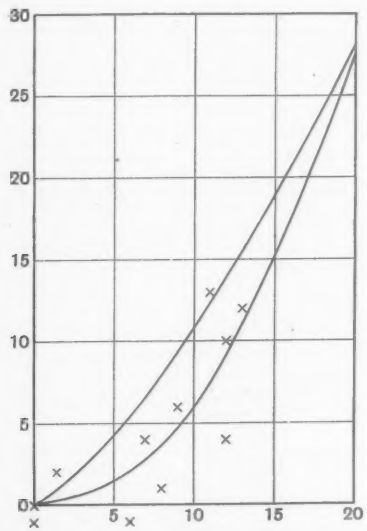

Fig. 3. Lower portion of dissociation curves of human blood. The upper curve is drawn from Douglas, Haldane and Haldane's formula, the lower curve from Hill's formula. Ordinate = percentage saturation with oxygen; abscissa = oxygen pressure in mm.

It may be taken as proved that either by accident or otherwise the curves for human blood at normal alveolar CO_2 pressure closely follow Hill's equation, which is founded upon a physical conception. The natural test is to see whether curves obtained under widely different conditions also agree with the formula.

The simplest way of varying the curves is to alter the concentration of the carbonic acid. In this way a whole series of curves may be produced as was shown by Bohr, Hasselbalch and Krogh [1904]. Poulton and I [1913] investigated the question of whether three curves of the series other than the normal curve at 40 mm. pressure could be represented by Hill's formula. The answer was striking for not only were the curves capable of expression by the general formula $y/100 = \frac{Kx^n}{1+Kx^n}$, but over the whole series only one of the two constants (K) varied, n remaining at a constant value throughout. Hence it is possible from a graph relating the concentration of carbonic acid to the value of K to obtain the dissociation curves for concentrations of carbonic acid intermediate between those at which the determinations were actually made. As this has proved useful on many occasions I append the diagram.

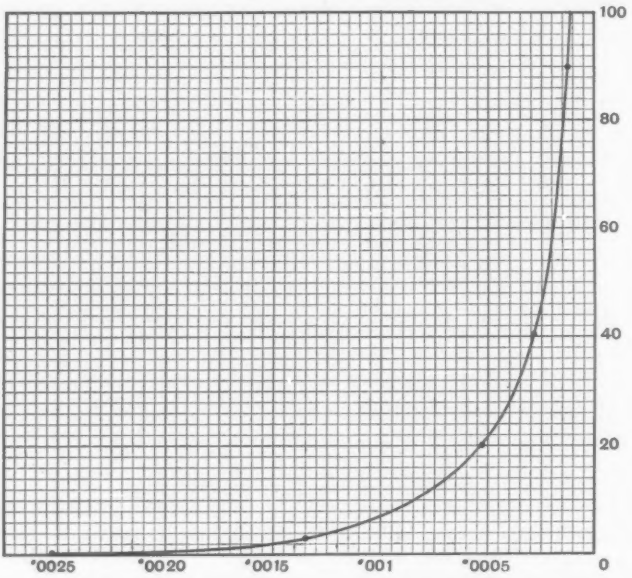

Fig. 4. Ordinate = CO_2 pressure in mm. Abscissa = K.

It was shown by Orbeli and me [1911] that carbonic acid had no specific effect in changing the affinity of haemoglobin for oxygen, but that it shared this property with lactic acid: Mathison subsequently showed that other organic and inorganic acids had a like effect.

The following points were obtained by the addition of lactic acid (0·2 %) to blood from which the carbonic acid had been as completely as possible removed by shaking.

Pressure of oxygen	10	20	20	30	30	40	50	50
Percentage saturation observed ...	11	39	40	64	68	82	87	86
Divergence from calculated results $n=2{\cdot}5$, $K=0{\cdot}000363$	+1	−1	0	0	+4	+2	+1	0

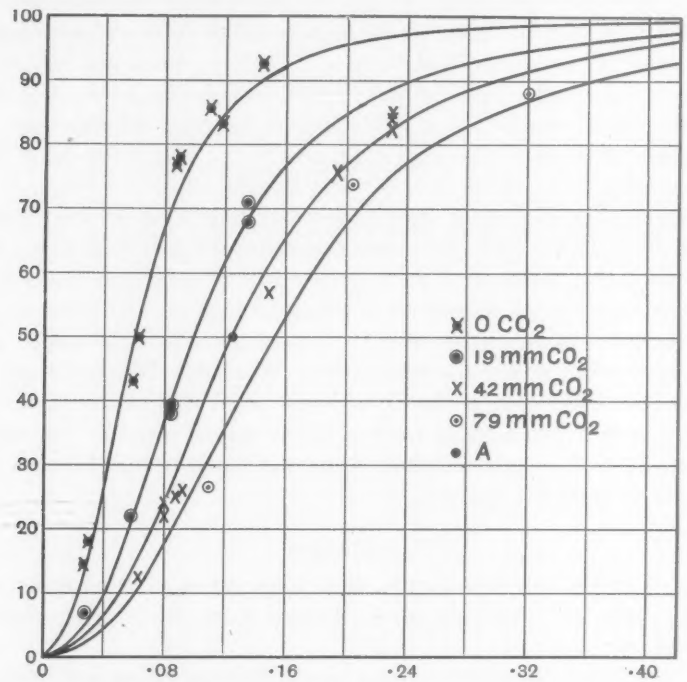

Fig. 5. Equilibrium curves of CO haemoglobin drawn from Hill's formula. The points are the observations of Douglas, Haldane and Haldane. Ordinate = percentage of haemoglobin with CO; abscissa = CO pressure in mm.

In addition to this, curves might be cited in which both lactic and carbonic acid were added to blood, such curves for example as those given by me in the record of my work in Teneriffe and by Orbeli and myself on the effects of low oxygen pressures on animals. The points on curves all follow the equation with the same degree of exactitude as those cited in this paper, but the points determined in each case were too few to make any detailed discussion of them worth while.

Most of what has already been said as regards oxyhaemoglobin curves might be repeated with regard to CO-haemoglobin. Fortunately a very complete series of data on this subject has recently been published by Douglas, Haldane and Haldane. In Fig. 5, these data are shown and along with them a system of curves drawn from Hill's formula. The scale on which the diagram is plotted is such that the centre point marked *A* of the curve corresponding to 40 mm. CO_2 would be on the same place in the figure as the centre point of the oxyhaemoglobin curve corresponding to the same CO_2 pressure. That being so it will be found that the whole figure is superposable upon the same figure for oxyhaemoglobin. Not only are the points then faithful to Hill's formula, but on a different scale the relation of *K* to the CO_2 pressure is the same in the case of oxy- as of CO-haemoglobin.

There is one more point to which I should draw attention, and which is involved in the fidelity of the observed points to the theoretical lines. Did the lines in Fig. 5 actually conform to Haldane's formula, there would have been the same slight discrepancy between the lines and the points at the bottom of the diagram in the case of CO-haemoglobin as in the case of oxy-haemoglobin and to just the same extent. When this difference reappears in another series such as the CO-series in which the points have been arrived at by wholly different methods (these were obtained by the method of carmine titration) the weight of evidence in favour of their having a real significance is greatly increased.

Conclusion.

1. The available data for the dissociation curves of blood agree very closely with the theoretical curves deduced from the following physical conceptions:

(*a*) That the reaction between haemoglobin and oxygen is a reversible chemical change $Hb_n + nO_2 \rightleftarrows Hb_nO_{2n}$.

(*b*) That *n* is the average number of molecules aggregated together, the value of *n* depending upon the nature and concentration of the electrolytes in the solution.

(*c*) That the effect of acids is to change the equilibrium constant of the reaction without sensibly altering the degree of aggregation of the molecules.

(*d*) That the above reaction does not involve the breakdown or reformation of the aggregates.

(*e*) That unsaturated oxides are unstable and break up into haemoglobin and saturated oxides.

2. The available data with regard to the reaction of CO and oxygen support an entirely similar conception of carboxyhaemoglobin.

3. So far as the curves deduced from the formula $y/100 = \frac{Kx^n}{1+Kx^n}$ (Hill) can be distinguished from those yielded by the formula

$$x = \frac{K_y[1+b(1-y)]}{(1-y)(1+ay)} \text{ (Douglas, Haldane and Haldane),}$$

the experimental evidence leans towards the former.

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XLIX. SEPARATION OF PROTEINS.
PART III. GLOBULINS[1].

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Up till the middle of last century it was generally held that the protein of blood serum and other kindred fluids was a single homogeneous substance —albumin. The word globulin, apparently first used by Berzelius, was applied by him to two substances, the protein part of haemoglobin (called by him haemato-globulin) and the protein of the lens, and came thus to be associated with cell-protein. That the serum protein could be split up, or that serum contained more than one protein was first suspected on account of some experiments of Liebig, Zimmerman and others, who found that great dilution of blood-serum produced a precipitate, especially after neutralisation with acetic acid. It was, however, Panum [1851] of Copenhagen who first discovered serum-globulin, and who developed the now classical method of preparation by first diluting the serum with water, and then adding acetic acid. Panum showed that the substance occurs constantly in human blood both in health and in disease, and called it serum casein from its resemblance to milk casein. Shortly after Zimmerman [1854] published the carbonic acid gas method of preparation. A few years later Alexander Schmidt [1862] in the course of his extensive observations on serum and kindred fluids showed in all cases that, side by side with the more soluble albumin, there was always the more insoluble globulin. In pursuance of his views on blood clotting he called the globulin in serum fibrino-plastic substance, though as often as not in his writings he calls it globulin.

Considerable activity followed on the publication of Schmidt's views: several ways of producing protein precipitates in serum were discovered and discussed; and there was some little confusion as to whether there was more than one substance, and as to nomenclature, till Heynsius [1869, 1876] gave

[1] Part I Haslam [1905], Part II Haslam, [1907].

good reasons for believing that, whether precipitated by simple dilution, carbonic acid gas, dilute acids, or saturation with sodium chloride there was only one substance—para-globulin as it was then called—a conclusion that, except in regard to sodium chloride, still holds good.

In 1878 Hammarsten [1878] published his great attempt at the purification and estimation of this substance. He introduced magnesium sulphate which produced a much larger precipitate than the reagents hitherto employed. In 1883 Burckhardt [1883], in repeating Hammarsten's experiment showed that, in addition to the globulin which was insoluble in water, a water-soluble substance was contained in the magnesium sulphate precipitate. Thus the probability of the existence of a third protein in serum was shown, though Hammarsten maintained that the magnesium sulphate precipitate was a single substance. Burckhardt's observation was, however, confirmed by Marcus [1899].

Meanwhile Hofmeister had conceived the idea of dividing serum and other liquids into fractions by means of taking precipitates at different degrees of concentration of one and the same salt solution, and thus isolating the different proteins. The first attempt was carried out by Kander [1886] when he precipitated globulin by half-saturation with ammonium sulphate, and albumin by complete saturation of the filtrate, the globulin roughly corresponding to Hammarsten's. Later on this system was elaborated by Pick [1902], Fuld and Spiro [1900], and others, both in the case of albumoses and serum. It was at first found that the globulin brought down at half-saturation could be split into two portions. The first was found to resemble the original water-insoluble substance of Panum, Schmidt, Heynsius and others, and was accordingly named eu-globulin; the second was more soluble and was called pseudo-globulin. In a further research Porges and Spiro [1903] thought there were three distinct fractions, both by ammonium, sodium, and magnesium sulphate; and Reiss [1904] also in Hofmeister's laboratory, decided on three—eu-globulin, pseudo-globulins I and II. Freund and Joachim [1902] on the other hand, taking the two fractions eu- and pseudo-globulin, showed that each contained a water-soluble portion, and took the view that there were four globulins. In regard to this system of fractionation I showed by direct experiment in Part I [1905] of this series that no single precipitation, both in the case of albumoses and serum proteins, whether by acid, salts of heavy metals, salting out or alcohol, ever produced a complete separation: that in the precipitate the substance of the filtrate could always be demonstrated, often up to 20 or 30 %; and similarly, mutatis mutandis, with the filtrate. And Wiener [1911], working on quantitative lines, showed that

reliable estimations of globulin could not be made by single precipitations. J. Mellanby [1907] from a determination of the percentage of protein at gradually increasing concentration of alcohol drew the conclusion that there were three different proteins in serum. Fractional methods, then, seemed to have left the subject in a more confused state than that in which they found it. I believe, however, and hope to show in these pages, that the method is inherently sound, and must for the present be regarded as one of the most important, and in many cases the only, method, we have of separating proteins.

Fractional precipitation.

The fundamental observation on which the method rests is that when a precipitant is added gradually to a protein fluid, and the resulting precipitate collected in successive portions, it is found that one portion differs from another. It is inferred from this that a separating process has been set up. And the proof of the correctness of this inference is found in the fact that if the process is continued sufficiently, substances that are undoubtedly distinct from each other can be obtained. Now in the case of the precipitant being a salt such as ammonium sulphate each increment of the salt in the protein fluid is followed by an increment of the precipitate. It is possible that at some concentrations the precipitate falls rather more thickly than at others: and indications may thus be afforded as to how a separation may be attempted. But it is entirely fallacious, as I have already pointed out, to suppose that one protein is wholly, or even nearly wholly precipitated before the next begins to come down. If there are two or more proteins their precipitation commences almost if not quite simultaneously, and they continue to come down together till the end of the precipitation, though at any one time different quantities of each might be coming down. Mellanby has demonstrated this point in the case of serum and ammonium and magnesium sulphate, having made quantitative experiments and plotted curves therefrom. He finds the precipitate falls very uniformly and that at no point is there a cessation of precipitation. His conclusion, however, that no splitting up of the serum can be brought about by these means, is erroneous. If no separation were brought about by the salting out, the protein in any one fraction would resemble that in every other. But this is obviously not the case. The chief cause of confusion hitherto has been of the opposite kind; that there has been no way of determining in the case of differing fractions, whether each fraction connotes a separate substance or not.

Let us suppose that there are two substance A and B; that A in a pure

state is precipitable more easily and is all precipitated at half[1] saturation; while *B*, in a pure state, does not commence to come down till the half-saturation point is reached, and only comes down completely on full saturation. Suppose now that three successive precipitates are taken at one-third, two-thirds, and full saturation. The portions would be composed as follows:

Fraction 1.	n_1 parts *A*,	m_3 parts *B*.
2.	n_2 parts *A*,	m_2 parts *B*.
3.	n_3 parts *A*,	m_1 parts *B*.

where $n_1 > n_2 > n_3$ and $m_1 > m_2 > m_3$.

Each fraction would thus show some difference from every other. Let us suppose that each fraction is redissolved and again precipitated at the same concentration. The three fractions would then differ from each other more markedly. The first would contain a greater proportion of *A*; the last would contain a greater proportion of *B*; while the middle one would remain about the same, much the greater part coming down between the precipitation limits. Generally speaking the separating power of the salt is not sufficient to cause any great change at any one precipitation. Thus far we might consider that we were dealing with three separate substances.

There are two principle methods by which to determine whether a fraction represents a substance or not.

1. Constancy of quantity under repetitions of the process. Fractions 1 and 3 would show this constancy after a time. Fraction 2 consisting of a mixture, would not, but would gradually disappear.

2. Subdivision of a fraction to find whether it is consistent or not. Fraction 2 on subdivision would show that it consisted of substances properly belonging to fractions 1 and 3. At half-saturation, in short, it can always be divided into a precipitate containing a higher proportion of *A* than *B*, and a filtrate containing more *B* than *A*.

Having decided on the number of substances into which the parent body can be split, there next arises the question as to how far each can be separated from the other. In the case of *A*, after a certain number of precipitations, the filtrates contain a constant quantity of organic nitrogen: that is to say, the separating process has entirely ceased. The separation, therefore, is presumably complete, but I will return to this point later. In regard to *B*, when we arrive at the point that half-saturation produces no precipitate, we cannot infer that the separation is complete. I have shown in previous

[1] As in previous papers I follow the usual convention of describing as "half" saturated a solution made by mixing equal volumes of saturated salt and protein solution. In reality such a solution is less than half saturated.

papers that, if a further small addition of salt be made, the resulting precipitate will consist largely of *A*; that is to say, a quantity of *A* remains dissolved in the *B* fraction. Means must be adopted, therefore, to continue the separation. Hitherto, the procedure has been to take a small fraction, dissolve it, and reprecipitate at the same concentration. The precipitate consisting mostly of *A*, is withdrawn, while the filtrate is returned to the main solution. This may be continued until no further trace of *A* can be found in the fractions. The separating process, then, comes to an end in this case also. But although the separating process has come to an end we cannot assume that the substances are completely separated, because in analogous separations in fractional distillation, crystallisation and precipitation it may happen that a certain amount of the substance being got rid of remains with the substance being purified, the two together, in this instance, acting as one substance towards the separating agent. Proof positive, therefore, can only be obtained by means of independent reactions. In the case of most proteins these, at present, are few in number: it is obvious that some separation must precede the discovery of typical reactions based on constitutional differences. In one of the separations to be described, we have such a reaction, and it goes far, I think, in demonstrating the validity of fractional precipitation in the case of serum proteins.

In regard to the question of the chemical individuality of the products of such separation, it can only be said that it is convenient to regard them as individuals until they are shown to be capable of further subdivision. Each can be tested with all the means at our command.

First Separation.

In the case of serum (ox-serum was used in all these experiments) my first procedure was to divide it by means of half-saturation with ammonium sulphate. The serum was diluted some four times with water, and an equal volume of the saturated salt solution was added. The resulting precipitate was collected, redissolved in water, and the process repeated until the separation, as shown by the sulphuric acid decomposition test[1], was complete. The separation was an easy one at this concentration; three or four precipitations got rid of all but a small quantity of albumin, and the

[1] 8–10 c.c. of the filtrate are mixed with an equal volume of concentrated sulphuric acid in a test-tube and warmed to boiling point. As the protein in the filtrate diminishes the tint gets lighter and when two successive filtrates give the same tint the amount of protein in the filtrates has become constant and the separation is at an end.

separation was completed in some six precipitations. The globulin fraction so obtained was almost entirely soluble in salt and water.

I then sought to divide it further by ammonium sulphate. Precipitation at one-third saturation (one volume of saturated solution being added to two volumes of the globulin solution) was carried out, and in this way the protein was divided into two fairly equal portions, the greater portion, perhaps, being that precipitated. The precipitate was re-dissolved and re-precipitated; and the process was repeated until the filtrate showed only small quantities of protein. During the progress of the precipitations, a larger and larger portion of the precipitate became insoluble in salt and water, and the experiment, on this account was not continued to a point of constancy in the usual way, since the insoluble matter might have held to itself soluble protein and so have vitiated the result. It served to indicate, however, that the globulin of half-saturation could be further divided into a water-insoluble part, precipitable at one-third saturation, and a more soluble portion not so precipitable. The latter was then treated by the process of fractional precipitation to remove any water-insoluble globulin that had remained dissolved in it. Powdered ammonium sulphate was added gradually and dissolved by stirring until a small precipitate, say some 15–20 % of the amount of protein present appeared. This was filtered off, redissolved in water, and saturated salt solution was added till the solution was at "one-third" saturation. This caused precipitation of a good proportion of the fraction. Examination showed it to consist largely of water-insoluble globulin. The filrate from the precipitation was returned to the main liquid, and the whole process was repeated some five times, the last fraction showing only quite a small amount of globulin. A further small amount of globulin was removed by dialysis.

The resulting substance was easily and completely soluble in water, and could be precipitated at half-saturation with ammonium sulphate. It was tested for albumin. A portion was dissolved in 50 c.c. water and precipitated by the addition of 50 c.c. saturated salt solution. This precipitate was redissolved and re-precipitated in the same way at the same volume[1].

90 c.c. of 1st filtrate gave by Kjeldahl 1·0 c.c. N/10 NH_3,
90 c.c. „ 2nd „ „ „ 0·95 c.c. „ „

The small amount of protein in the filtrate, being constant in quantity, is presumably pseudo-globulin.

It was mostly precipitable by saturation with sodium chloride and nearly completely by magnesium sulphate. With ammonium sulphate most of it

[1] For details of this method see Haslam [1905].

(say five-sixths) was precipitated by the addition of only three-quarters of a volume of saturated solution (43 % saturation). Half-saturation precipitated all but traces. This, then, was the pseudo-globulin of Hofmeister, the existence of which was indicated by the experiments of Burckhardt and Marcus. Its solubilities are intermediate between those of the water-insoluble globulin and albumin.

I next proceeded to ascertain whether this substance could be split up further by ammonium sulphate. Freund and Joachim found that the pseudo-globulin of Hofmeister was partly soluble, partly insoluble in water: but the insoluble portion was probably merely globulin left dissolved, as we have seen, in the pseudo-globulin. It was possible, however, that it could be split into two soluble bodies after Spiro and Reiss. A portion was divided into two roughly equal parts by ammonium sulphate in the following way. A dilute solution (about 0·25 %) was made. A small test portion was completely saturated with salt and placed in a cylindrical beaker. Saturated salt solution was then added to the main liquid until a small amount, double the volume of the test portion, placed in an exactly similar cylindrical beaker, showed on looking downwards the same degree of density of precipitate. I then assumed that about half the protein was precipitated. This, after being allowed to stand, was filtered off and the filtrate saturated to obtain the remainder. These two fractions closely resembled each other in their solubilities. Each was largely precipitable on the addition of three-quarters its volume of saturated salt solution, and the portions left over in solution appeared in no way different from those precipitated. Nor did further treatment on fractional lines show that the substance could be divided.

Since we had a water-soluble globulin commencing to fall at 33 % saturation, and nearly finishing at 43 %, that is to say when free or nearly free from other proteins, it might be supposed that a substance existed that commenced to fall at 43 % and was mostly precipitated at 50 % saturation. It might be supposed that if such a substance existed it might be nearly or entirely lost in the series of precipitations at 50 % in which albumin is got rid of. To test this point, I began afresh with some serum and endeavoured by suitable fractionations to find such a body; but entirely without success.

Experiment. 300 c.c. ox-serum were taken and diluted to 1000 c.c. and 500 c.c. saturated salt solution were added to get rid of some of the globulin. To the filtrate were added some 700 c.c. (over half-saturation) further of saturated salt solution, so that most of the searched for substance should be precipitated while as much albumin as possible should be left in the filtrate. The precipitate was dissolved again in 1000 c.c. water and the above

precipitations were repeated. The protein resulting was then dissolved in 900 c.c. water and 500 c.c. of saturated salt solution were added so that not only globulin but pseudo-globulin might be largely precipitated also. To the filtrate was added salt solution again to make rather more than half-saturation. I proceeded in this way to eliminate gradually portions of globulin and pseudo-globulin, on the one side, and albumin, on the other; always being careful to work well outside the precipitation limits of the body for which I was looking, so that as little as possible might be lost. No such substance, however, could be found. The process was continued until a quantity too small to work with remained, and up to the last, pseudo-globulin on the one hand and albumin on the other could be demonstrated. This experiment was repeated on another sample of ox-serum.

I concluded, therefore, that no substance exists in ox-serum precipitable between 43 and 50 % salt concentration, at any rate in quantities comparable to globulin and albumin.

Water-insoluble globulin.

As it was not possible to carry to completion the separation of globulin by means of ammonium sulphate, owing to its becoming increasingly insoluble in the salt solution, I tried precipitations with acetic acid. Some water-insoluble globulin that had been prepared from ox-serum by a few precipitations at one-third saturation and then diluting with water, was shaken well with water, thrown on the filter, and washed with water. It was then suspended in water, and a few drops of ammonia were added. The globulin completely dissolved. The volume of the solution was 400 c.c. About 0·65 g. of globulin was recovered at the end of the experiment, so that if we allow for some 20 % of other proteins and loss during the experiment, there would be some 0·8 g. protein. Thus the strength of the solution was about 0·2 %.

The globulin was then precipitated by the addition of dilute acetic acid. The precipitation began when the solution was about neutral, and was rapidly completed on the addition of further acid, the solution being finally faintly acid. The mixture was allowed to stand over-night. The precipitate was then filtered off, suspended in water, and redissolved by the addition of ammonia. Normal solutions of ammonia and acetic acid were used, the quantities required being added out of burettes. Before precipitation the volume of the solution was always made to 400 c.c. 16 c.c. alkali were used to dissolve: the precipitate was usually complete on the addition of some

13 to 14 c.c. of acid, but 16 were added. On one occasion a small excess was added, but no re-solution of the precipitate occurred. It was noted that the globulin took more time to dissolve as the experiment advanced.

In the second filtrate the presence of water-soluble protein was easily demonstrated. The fourth and fifth filtrate each showed a faint cloud on boiling, and a faint precipitate on saturation with salt. In each, however, by saturating the whole with salt, and collecting the small quantity of protein obtained, water-soluble protein could be demonstrated. The eighth and tenth filtrates were examined quantitatively for organic nitrogen by the method described in previous papers.

Eighth filtrate volume 345 c.c. gave 2·4 mg. N.
Tenth ,, ,, 343 c.c. ,, 2·4 ,,

Thus in precipitating the water-insoluble globulin by acetic acid a point can be arrived at in which the organic nitrogen in the filtrate is constant and the soluble pseudo-globulin presumably completely eliminated. Reckoning that globulin contains 16 % N, the amount in these filtrates would be 0·0073 g. or 0·004 %. It may be noted that the separation of globulin from pseudo-globulin is considerably more difficult than the separation of pseudo-globulin from albumin.

Physical Changes caused by Separation.

The substance thus obtained is distinctly less soluble than that prepared more rapidly by a smaller number of precipitations. Some of the essential peculiarities of globulin are, however, preserved. The addition of alkali causes it to swell up into a jelly-like consistence. It is, on the other hand, only slightly soluble in salt solutions. But the question of the alteration of the physical properties of proteins on precipitation is a difficult one.

Globulin prepared by a single precipitation with acetic acid from diluted serum is a comparatively soluble substance. It also has mixed with it considerable quantities of pseudo-globulin and albumin. The more it is precipitated, the more insoluble does it become, and it is very generally believed that this is due to a physical alteration. But it is not necessary to postulate physical alteration, at any rate, to the degree generally done. Globulin can be held in solution by the other proteins of serum. This can be shown by taking a portion of serum-protein, by precipitating with ammonium sulphate at half-saturation twice or thrice and dialysing until all the salt is removed. A certain amount of globulin will be precipitated; but no matter how long the clear solution from which the precipitate may be

removed is allowed to dialyse, water-insoluble protein in large quantity can be demonstrated therein. Or, more simply, serum may be dialysed indefinitely and after filtering off the resulting precipitate, large quantities of globulin can be found in the filtrate. Again, during the course of fractional experiments, globulin is commonly found in solutions half-saturated and more than half-saturated with ammonium sulphate, there being excess of other protein present. Indeed, it is this property that makes the whole difficulty of the separation. It is to get rid of the globulin that remains dissolved in the pseudo-globulin that the many precipitations at high salt concentration have to be resorted to. It is this property that makes it impossible by any one precipitation by any agent to obtain more than a proportion of the globulin present. Pseudo-globulin and albumin are, in fact, the chief solvents of globulin in serum. But in this case it is clear that in any series of precipitations for its preparation and purification, where it loses at each precipitation a portion of the other protein, it loses a solvent. The more the other proteins are withdrawn from it, the more insoluble must the preparation become. Quite apart, therefore, from any changes that may go on in the globulin as a result of the physical action of the reagent used, we see that any preparation must become more insoluble as it becomes purer. But there is little doubt that some of the various reagents used may also cause insolubility. This is evident from the different behaviour of globulin when precipitated by acetic acid or ammonium sulphate. With ammonium sulphate, as we have seen, globulin gradually becomes so insoluble as to resemble coagulated protein. It may be noted that this change does not occur uniformly throughout the globulin, but a small portion of such insoluble matter appears, and continues to increase as the precipitations proceed. We may therefore argue that, apart from its separating power, ammonium sulphate has an action on globulin tending to render it insoluble. With acetic acid such action is extremely small; after many precipitations only quite a small proportion of this very insoluble matter appears.

But although it is quite easy to prepare solutions in which globulin is held in solution by the other proteins, it is apparently difficult to reproduce this condition when the various proteins have been separated from each other. At any rate, it cannot be produced by the simple addition of globulin to a solution of albumin or pseudo-globulin. I have no quantitative experiments on this point, but it is quite clear that only small quantities, if any, of completely separated globulin dissolve directly in watery solutions of separated albumin or pseudo-globulin. So far as my experiments have gone, it seems to be the case that, after a small degree of separation, say two or

three precipitations, the component parts can be restored to the *status quo*; but that the farther the separation is pushed, the more difficult does the restoration become. This would seem to show that each act of separation causes some secondary molecular change in the parts separated.

In this connection I may recall the remarkable fact to which Hardy draws attention: that while serum itself can be readily filtered through a porous pot, globulin, even in the early stages of preparation, cannot. Hardy also takes occasion to point out that neither alkali nor salt is capable of producing so high a grade of solution as that of globulin as it exists in serum, and that a further dissolving agent must be present. To say that that agent is the other serum protein is not very different from saying that there is some sort of combination among them; a conclusion to which Hardy is led on other grounds. In the first paper of this series I brought forward some evidence that this seems also to be the case with the albumoses.

Allowing, however, for the greatest amount of change in the process of separation, it can hardly be supposed that any radical change of constitution occurs: the changes are extra- rather than intra-molecular. So that from the point of view of chemical analysis, the matter is not of great importance.

Possibility of Substances between Globulin and Pseudo-Globulin.

In the series of precipitations at one-third saturation, it was found that, even after seven or eight precipitations at sufficient dilution, the product obtained was largely soluble in water. After dialysis a good proportion, perhaps 50 %, remained in solution. The clear solution certainly contained some pseudo-globulin, and might well have consisted entirely of this and globulin. We have seen that pseudo-globulin cannot be further split up, but there was a possibility that the bulk of the soluble matter consisted of a third substance, more soluble than globulin, but precipitable at one-third precipitation with the globulin. To ascertain whether such a substance existed, experiments were conducted on the following lines. Diluted serum is precipitated some eight or nine times at one-third saturation: this gets rid of all albumin, and a considerable quantity of pseudo-globulin. Globulin is then removed by fractional precipitation, care being taken to remove only insoluble matter, all doubtful portions being returned to the main solution, so that the quantity of substance sought for should not be diminished in this way.

This leaves us with a more soluble substance again, and from this, more pseudo-globulin is removed by precipitation at one-third saturation.

Thus alternately portions of globulin and pseudo-globulin are removed

from the protein under examination. This can be continued until the substance sought for is found, or there is no more substance to work with.

Two separate experiments were carried out, and on several occasions portions of protein were obtained which were precipitable at "one-third" saturation, and soluble in water—further examination, however, always showed them to consist of globulin and pseudo-globulin. I select the following for quotation.

500 c.c. ox-serum were diluted to 2,000 c.c., and a precipitate formed by the addition of 2,000 c.c. saturated salt solution. The precipitate was dissolved in 2,000 c.c. and a further precipitate formed by the addition this time of 1,000 c.c. saturated salt solution (one-third saturation). This latter precipitation was repeated eight times when the filtrates were found to contain only quite small quantities of protein. During the series of precipitations large quantities of insoluble matter appeared, and were removed from time to time. The final precipitate contained a fair proportion of protein that was soluble in water. From this I sought to remove any water-insoluble globulin that it might contain by means of the system of fractional precipitation before described. The protein was dissolved in 200 c.c. water (the first series of precipitations had considerably reduced it in bulk), and some 60 c.c. saturated solution were added. The small precipitate that fell was found to consist largely of water-insoluble globulin, but mixed with it was a certain amount of water-soluble substance, and this was returned to the main solution. To this a further quantity of salt was added and another fraction obtained, which was treated in the same way. Some six fractions were taken and in the last two only quite small quantities of water-insoluble matter were found. The experiment was accordingly brought to an end by nearly complete saturation of the liquid, and the resulting protein was examined. On being dissolved in 80 c.c. of water it was found that on addition of 40 c.c. saturated salt solution (one-third saturation), only some two-thirds of it were precipitated. The remainder came down on half-saturation. It was clear, therefore, that pseudo-globulin was present to a considerable extent. A fresh series of precipitations at one-third saturation was therefore undertaken, some eight in number. The resulting substance of which there was only a small quantity, was again found to contain both water-insoluble and water-soluble parts. Owing to the withdrawal of a large quantity of pseudo-globulin, the insoluble globulin was easily demonstrated again. The water-soluble part, after further precipitations of a similar character to those already described, was shown to contain both water-insoluble globulin and pseudo-globulin.

It was thus seen that no body precipitable at one-third saturation and at the same time water-soluble, could be obtained that remained constant in quantity, and could not be resolved into globulin and pseudo-globulin. Such fractions, therefore, must be looked upon as mixtures. Or if we look upon the serum proteins as one molecular structure in the serum, such fractions must be regarded as portions of that molecular structure which have so far resisted the disintegrating action of the salt; but which, by further action can be resolved into globulin and pseudo-globulin. And it may be further remarked that by precipitation at suitable concentrations fractions of material or portions of the original molecular aggregate may be obtained having any required solubility, and exhibiting a certain appearance of constancy. This is much more the case with mixtures of globulin and pseudo-globulin than with those containing albumin. The two former are much more closely connected at any rate in regard to their behaviour to salt, than are pseudo-globulin and albumin. As we have already seen, albumin can be removed comparatively readily.

I thus arrived at the general conclusion that there are two, and only two different proteins that are precipitable at half-saturation with ammonium sulphate; the historic water-insoluble globulin and the protein soluble in water corresponding to Hofmeister's pseudo-globulin. In addition to the precipitation differences between these two bodies, there are also certain differences in appearance. The soluble substance comes down in finer particles and has not the flocculence of the globulin precipitate. It is perhaps rather whiter and does not become discoloured so readily as globulin.

The Separation and Purification of Pseudo-Globulin.

We are now in a position to discuss the separation and purification of pseudo-globulin in greater detail, with a view of determining the limits of fractional methods, and the most convenient way of applying those methods to this particular case.

To obtain crude pseudo-globulin for further experiment, diluted ox-serum was taken; globulin and albumin got rid of at one-third and half-saturation respectively. After some two to three repetitions, a sample of pseudo-globulin can be obtained, giving no precipitate at one-third saturation, and leaving only traces of protein in the filtrate at half saturation. I will now describe the method of fractional precipitation I have hitherto employed in greater detail.

The crude protein, in this case pseudo-globulin, is dissolved in water and saturated salt solution is added until a precipitate begins to fall. This

is usually just after one-third saturation. In the early stages of the experiment a good precipitate is obtained at "one-third" saturation of the fraction. After the first few removals of globulin, however, this is no longer the case. The concentration of protein in the solution at this point may be 2–0·5 %. The readiest way of determining this is by a Kjeldahl determination of the total organic nitrogen, calculating the protein as containing 16 % N. The precipitate formed may be some 20 to 25 % of the whole quantity of the protein present. This is determined by comparing the opacity of the precipitate formed with that in a small quantity of the solution in which the protein has been completely precipitated by the addition of solid salt as has been described before. In using this method it must be remembered that the precipitate continues to fall gradually for some hours after the addition of a quantity of salt, so that only a rough determination can be made at the time of adding salt. The rate of fall of the precipitate appears to depend, to some extent, on the concentration of the protein: the diluter the solution the slower the fall. After the formation of the precipitate the solution is allowed to stand 24 hours. If the precipitate has sunk to the bottom, most of the liquid can be decanted. The remainder is filtered and the precipitate is redissolved. Some 35–30 % of this is then precipitated at about the same concentration as in the previous experiment.

This precipitate being some 9–6 % of the total protein present, is removed, and the filtrate is added to the main solution. The amounts withdrawn are gradually lessened as the experiment is repeated and may be tested for the protein it is desired to eliminate, in this case dialysis being used to detect globulin.

The process can be repeated indefinitely. When, however, the separation proceeds slowly, that is, when at each precipitation the proportions of the substances to be separated do not differ much in the precipitate and filtrate, it is expensive both of material and time. If the fractions are large the material is soon used up, and if small the process becomes very slow.

The amount of the fraction should correspond to some extent with the amount of protein present which it is desired to eliminate. As will be seen from the experiment quoted, some six or eight repetitions of this process will reduce the insoluble globulin in pseudo-globulin from 8–10 % to 2–3 %. The volume of the solution is increased at the end of each pair of precipitations. When it becomes inconveniently large it can be reduced by salting out the protein completely from a portion, dissolving the protein so obtained in a minimum of water, and returning it to the main solution.

In employing this method in the separation of the albumoses in some

cases I had no means of determining the progress of the separation, beyond that of noting the increasing solubility of the protein being purified and obtaining from successive fractions diminishing quantities of the more insoluble protein. Where hetero-albumose was being salted out there was the more independent test of precipitation by dialysis. This is the case in the present instance. This test, however, is only a solubility one, and cannot be relied on in the same way as some independent reaction. In the present case we have this latter: since, while globulin contains phosphorus, pseudo-globulin does not contain any. Globulin only contains about 0·1 % P, so that in spite of the possibility of detecting very minute quantities of phosphorus the test is limited in its usefulness. It is sufficient, however, to show the general course of the separation, and to enable us to place a value on the solubility tests.

Hardy [1905] first pointed out that globulin contained phosphorus and that other fractions of serum-protein containing less globulin contained less phosphorus. The relationship of phosphorus to globulin is not quite simple as will be pointed out more fully later on. It is sufficient for present purposes to note that salting out operations and dialysis apparently leave the phosphorus content of globulin unchanged; while samples of pseudo-globulin can be obtained which, by the most careful tests, give no phosphorus.

For detecting and roughly estimating minute quantities of phosphorus the ammonium phosphomolybdate method of Neumann was used; the decomposition of the protein being effected by Bayliss and Plimmer's modification to avoid using more sulphuric acid than is necessary. Ammonium phosphomolybdate contains only some 1·6 % P, and as very small quantities can be precipitated and detected, it forms, under proper conditions, a very delicate test. Further, owing to the way in which minute quantities of the precipitate fall, it is possible, simply by inspection, to estimate the amounts with rough accuracy.

Taking known quantities of a solution of sodium phosphate I have found that 0·005 mg. P can certainly be detected, sometimes 0·0025 mg. P. And if the conditions as to total volume of solution, quantities of reagents present, and heat used to effect precipitation, are maintained equally, the differences between such quantities as 0·005, 0·0075, 0·01, 0·015, and 0·02 mg. P, can be readily appreciated; and, by comparison, a rough estimate can be formed of the trace of phosphorus under consideration.

Good results were obtained with solutions differing slightly from Neumann's:

30 c.c. Water.
30 c.c. 50 % ammonium nitrate solution.
30 c.c. 3 % ammonium molybdate solution.
Heat to 85° C.

Experiments were also made to determine how far Neumann's method could be used to estimate small quantities of phosphorus. Using a decinormal in place of a seminormal standard solution, I found that the method did not lose in accuracy until the quantities were below 0·2 mg. P, when the estimations became too high. Below 0·1 mg. P (which corresponds to 0·9 c.c. decinormal solution) the values were quite unreliable:

(1)	0·52 mg. P	gave	4·8 c.c.	N/10	solution...	0·539 mg. P
(2)	0·26 ,,	,,	2·5 ,,	,,	...	0·275 ,,
(3)	0·26 ,,	,,	2·5 ,,	,,	...	0·275 ,,
(4)	0·208 ,,	,,	2·1 ,,	,,	...	0·231 ,,
(5)	0·208 ,,	,,	2·0 ,,	,,	...	0·22 ,,
(6)	0·104 ,,	,,	1·1 ,,	,,	...	0·121 ,,
(7)	0·104 ,,	,,	1·0 ,,	,,	...	0·11 ,,
(8)	0·104 ,,	,,	1·15 ,,	,,	...	0·126 ,,
(9)	0·078 ,,	,,	0·9 ,,	,,	...	0·099 ,,
(10)	0·078 ,,	,,	0·8 ,,	,,	...	0·088 ,,
(11)	0·052 ,,	,,	0·65 ,,	,,	...	0·0675 ,,
(12)	0·052 ,,	,,	0·8 ,,	,,	...	0·088 ,,

For the experiments about to be described some three different samples of ox-serum (each of about one to two litres) were mixed. In order to determine the amount of phosphorus in the globulin of this mixture of serums, some globulin was separated and purified, first by salt precipitation, and then by acetic acid in the way previously described.

200 c.c. of 7th filtrate gave 1·2 c.c. N/10 NH_3.
200 ,, 8th ,, ,, 1·1 ,, ,,

The globulin was washed on the filter paper until no more acid was shown in the filtrates and then washed with alcohol and ether. It was dried at 110° to constant weight.

1·0965 g. globulin gave 1·2055 mg. P = 0·11 % (by Neumann).
0·732 ,, ,, 0·785 ,, = 0·107 %.
Mean = 0·1085 %.

Therefore, 1 mg. P indicates 0·921 g. globulin.

I. Crude pseudo-globulin, giving no precipitate at one-third saturation and containing traces only of albumin. To determine the amount of globulin contained in it phosphorus was estimated by Neumann's method.

2·172 g. gave 0·19 mg. P corresponding to 8·4 % globulin.

The fractional process described above was then carried out six times in succession; large fractions were taken, some 28 % in the first instance and

35–40 % in the second. There was a marked increase in the solubility of each successive fraction, until, in the last fraction removed, I was unable, even after four sub-fractionations, to find any protein insoluble at one-third saturation. On the other hand, the fraction gave a distinct, though small, precipitate on dialysis. The experiment was terminated by salting out all the protein. To get rid of the small amount of albumin it was redissolved in water and thrice precipitated at half-saturation.

200 c.c. of 2nd filtrate gave 2·4 c.c. N/10 NH_3.
200 ,, 3rd ,, ,, 2·3 ,, ,,

Thus albumin was eliminated.

On dialysis of a portion a good cloud appeared. The whole amount of protein was precipitated by the addition of alcohol in the cold (below 15° as advised by Mellanby) and washed with alcohol and ether. A portion was dried at 110° to constant weight and the phosphorus estimated:

(1) 0·4115 g. gave 0·0125 mg. P by inspection...2·8 % globulin.
(2) 0·442 ,, 0·015 ,, ,, ...3·1 % ,,

If we take a mean and allow 20 % error, the amount of globulin in the sample is from 2·3–3·5 %.

The great contrast between the solubility in salt and the precipitability on dialysis was surprising and will be referred to later. This inconsistency was no doubt the basis of the observation by Freund and Joachim, who found that the fraction which gave no precipitate of globulin at one-third saturation gave a precipitate on dialysis so large that it was considered another substance.

II. Crude pseudo-globulin as in I. This experiment was on a much larger scale, and the process was repeated 36 times. The fractions were smaller than in I, being at first 20 %, but dropping later to 12–15 %. The dilution varied from 1–0·5 % in the earlier stages to 0·2–0·1 % in the later.

The salt solubility of succeeding fractions was noted, and a general agreement with the results in I was found.

After the ninth and twentieth fractions, the whole protein was salted out and dried. On each occasion it was found on re-solution that the solubility had decreased, and further that fractions taken immediately after re-solution were less soluble. The tenth fraction had the same solubility as the fifth, and the twenty-first as the fifteenth.

All the sub-fractions were tested by dialysis. It was sought by this means to follow the progress of the separation. The quantity of protein precipitated showed in general a decline. The decline was not a regular one, however. The first nine tests showed a steady decrease of substance. The tenth (after

drying) showed an increase, and from this to the seventeenth a slower and more irregular decline was noted. The twenty-fifth fraction showed no precipitate on dialysis, but the sub-fraction gave a cloud. The thirtieth fraction and sub-fraction remained clear. The thirty-first sub-fraction, however, gave a good cloud and the fraction a faint cloud. The thirty-fourth fraction and sub-fraction were again clear and also the thirty-fifth.

In general it was noted that when the fractions taken were small, the separation seemed to make very little progress. Observations were made as to the composition of the precipitates on dialysis, since in some cases they appeared out of proportion to the amounts of globulin that could be present. It was found that a considerable amount of pseudo-globulin was precipitated with the globulin, as the experiment became more advanced. The precipitate caused by the dialysis of serum or of the mixture of proteins obtained by one or two precipitations by "salting out" consists mostly of globulin, a certain proportion of pseudo-globulin and a small amount of albumin. The earlier fractions on dialysis gave such precipitates. In the later fractions, after the twelfth, it was found that on dialysis a large quantity of pseudo-globulin was precipitated with the globulin—in some cases an excess of pseudo-globulin. In one case where some 6–7 % of the protein present was estimated to be globulin, the precipitate was some 15–20 % of the protein present. Allowing, therefore, for 2 % globulin remaining in solution, the precipitate must have contained some 70–80 % pseudo-globulin, to 20–30 % globulin. Without attaching too much importance to these estimates, it is clear that considerably more pseudo-globulin than globulin was in this precipitate.

Precipitates of this kind remain insoluble, or nearly insoluble in water. They may be largely dissolved by the addition of salt, or better, a trace of alkali. In the latter case, if the solution be neutralised, no reprecipitation occurs, and further, if the solution be then dialysed there may be only quite a small proportion of the protein precipitated, or even none at all. This latter result could be attributed to traces of acid or alkali being left over after imperfect neutralisation. This was shown to be the case in many fractions containing small quantities of globulin. A portion dissolved in water and dialysed gave a small precipitate. Another portion which had been dissolved in dilute ammonia and then neutralised gave no precipitate on dialysis: nor did subsequent additions of traces of acid or alkali, nor prolonged dialysis, have any effect whatever in producing a precipitate.

The size and formation of the precipitate on dialysis appeared also to be connected with the rate at which the salt was withdrawn from the solution. In the majority of the experiments the precipitate reached its maximum in

24 hours; although there was then further salt in the solution, further dialysis produced no precipitate, even though continued many days. If, however, the solution was diluted four or five times with distilled water, a further precipitation was sometimes obtained.

It was also frequently noted that if pseudo-globulin containing a small proportion of globulin were dried, the precipitate on subsequent dialysis was increased. Both globulin and pseudo-globulin are rendered more insoluble by drying: by repeated drying both may be rendered entirely insoluble. So that the physical condition of pseudo-globulin would seem to be an important factor in inducing its precipitation with globulin on dialysis, and may have been one of the causes of the increase in the amount carried down with globulin already referred to.

It was also noted that, as the experiment advanced, a tendency to mechanical coagulation developed. This was chiefly noted in filtering, after a fraction had been precipitated. The protein in the solution then was just on the point of being precipitated. Unless the filtration was conducted slowly, small flakes and strings of protein appeared, which tended to adhere together at the end of the filter.

Towards the end of the experiment, also, the fractions were more affected by drying than in the early stages. Thus there is distinct evidence that the prolonged contact with salt induces slow physical changes in pseudo-globulin.

Only one intermediate observation of the amount of phosphorus was made. After nine fractions a portion was freed from salt, and precipitated by alcohol and dried.

0·347 g. gave 0·01 mg. P by inspection.....2·6 % globulin.

This shows a rather slower rate of progress than in I.

Of the final substance 0·561 g. was tested for phosphorus with a negative result.

The physical changes noted during the experiment were:

1. A gradual increase of solubility in salt solutions which, after a time, appears to cease. In no case did the increase go so far that pseudo-globulin became soluble in half-saturated, or nearly half-saturated solutions (that is at ordinary dilutions, 5–0·2 %).

2. The substance gradually became more liable to pass into the insoluble or coagulated state.

In regard to the dialysis test it was found:

1. That the amount of pseudo-globulin carried down with globulin on dialysis increases as the experiment proceeds; the pseudo-globulin, after a time, being in excess of the globulin.

2. That if the substance be dried before dialysis a considerably larger precipitate results.

3. That the amount and constitution of the precipitate is also dependent on various chemical conditions.

In regard to the phosphorus estimations, assuming that 0·005 mg. P is the smallest amount that can be detected, the final result would mean that the pseudo-globulin might contain a quantity less than 0·00087 % P, but not more. This, together with the rather better result in III, where nearly a gram gave a negative result, is practically conclusive that pseudo-globulin does not contain phosphorus. We can, therefore, attribute the small and diminishing amounts of phosphorus found in the experiments to the presence of globulin, and so far as we can estimate the phosphorus we can estimate the globulin.

Experiments I and II, therefore, show:

1. That by the method described all but some 2–3 % of the globulin can be eliminated in some six or eight fractionations.

2. That the larger the fractions taken, the more rapid the separation. So that the method becomes too lengthy if used economically as regards material.

3. That both solubility tests as guides to the progress of the separation are fallacious.

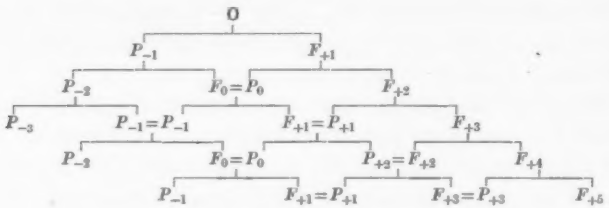
As to the progress of the separation in II after the ninth precipitation, it is clear from the negative result in the final test for phosphorus, that most of the remaining 2·6 % of globulin is eliminated. It would seem probable that the elimination is a very gradual one.

In the albumose separation I relied on solubility tests, especially the salt solubility one. In no case, however, did the indications point to such a rapid conclusion of the separation as in I: nor were such large divergences as those between I and II met with. Although, therefore, there may have been some small alterations in solubilities which would tend to falsify results, it is hardly possible that it could have been so extensive as in the present case. In all respects the albumoses showed themselves more stable under treatment than the serum protein.

Finally, I tried a different method with a view of obtaining greater rapidity. In place of taking only a small proportion of protein at each precipitation, which is only efficient when a comparatively large proportion of globulin is contained therein, I tried the following plan which is based on the principle of dividing the protein into two equal parts at each precipitation.

III. Some 10 g. of crude pseudo-globulin which had been fractioned

four times on the method described above, and contained about 4 % of globulin, were dissolved in 2,000 c.c. water. By the addition of solid salt about half the amount of protein as estimated in the way above described of comparing opacities was precipitated. The precipitate P_{-1} was dissolved in 2,000 c.c., and again by solid salt equally divided into P_{-2} and F_0. The filtrate F_{+1} was further divided equally as far as possible into P_0 and F_{+2} by the further addition of salt. At this stage, there were four divisions each containing about a quarter of the original protein. The process can be best explained by means of a diagram.



F_0 and P_0 were mixed being considered nearly of the same grade in purity; the precipitate being dissolved in a minimum of water and added to the filtrate. The mixture was then precipitated by the addition of solid salt as before and divided into P_{-1} and F_{+1}.

At each precipitation the globulin is divided into two portions, most going into the precipitate; so that the amount of globulin in the filtrates on the right F_{+1}, F_{+2} and F_{+3} is constantly diminishing. The process which involved a considerable number of precipitations was continued until a sufficiency of material of grades 3, 4, and 5 was obtained. It was found that an exactly equal division of the protein in any given experiment could not be obtained without an undue amount of time being spent. The method used was that already described of comparing opacities of precipitates. Owing, however, to the gradual fall of the precipitate which is the more accentuated the more dilute the solution, the precipitate cannot properly be estimated until twenty-four hours have elapsed. To save time, therefore, I endeavoured to allow for the extra fall on standing, by precipitating only some 35 or 40 % as estimated shortly after adding the salt. This led to several inexactitudes and probably accounts, in part, for the slight inequality of the results.

The phosphorus estimations were all done at the same time, and compared with each other as well as with known amounts:

Parent substance	0·335 g.	gave	0·015 mg. P...4·1 % globulin.	(By inspection)
Third grade	0·511 ,,		0·0075 ,, ...1·3 % ,,	,,
Fourth grade	0·867 ,,		0·0075 ,, ...0·79 % ,,	,,
Fifth grade	0·883 ,,		negative, *i.e.* contains less than 0·5 % globulin.	

The figures, allowing for the roughness of method of estimation, are sufficient to show that this method is more suited to eliminate small quantities than the other. It also has the advantage of economising time and material, as the precipitations, though numerous, can be carried on simultaneously, after the first few; and all material in various grades can be labelled and kept ready for use at any time.

These estimations of phosphorus, restricted as they have been owing to the small amount present, are, I think, sufficient to establish the main point that the separation of these two proteins can be completed, or nearly completed, by means of fractional precipitations.

In regard to the practical point of the preparation of pseudo-globulin containing, say, not more than 1 % globulin, I do not think any very extensive procedure will be necessary. I found that in precipitating pseudo-globulin containing some 2–3 % of globulin with alcohol in the cold, that a small fraction could be obtained which was comparatively rich in globulin. I have not concluded my experiments on this point, but it is not unlikely that after a few fractions with salt have got rid of all but some 3 % or so of the globulin, that a large part of the latter can then be eliminated by a few fractions with alcohol.

The Preservation of Globulin and Pseudo-Globulin.

I have already pointed out that both globulin and pseudo-globulin on being dried, at any rate with salt, become more insoluble. To keep them without drying I have found that, by the addition of a little ether to their solutions in dilute saline, they will keep for considerable periods in stoppered bottles. The solutions should be well shaken with the ether.

I have samples which I have kept over a year which are quite clear and show no tendency to precipitate.

Relation of Phosphorus to Globulin.

In the preceding section I have assumed that globulin contained phosphorus as an integral part of itself. This was because throughout such reactions as there described the globulin always retains phosphorus. It is difficult to believe that if the phosphorus could be split off by salt, ammonia,

or acetic acid, that any except a trace would have remained with the globulin after the prolonged operations entailed by its purification. However, to obtain further assurance I estimated the phosphorus in a sample of crude globulin prepared from serum by four precipitations of salting out at one-third saturation, followed by two by acetic acid, after solution in ammonia.

1·4725 g. globulin gave 1·462 mg. P...0·099 %.

This is distinctly less than in the case of the more purified globulin although it had suffered less than half the number of precipitations.

I have already quoted (page 507) the phosphorus determinations in one sample of purified globulin. Another sample from ox-serum was analysed:

0·5495 g. globulin; 0·575 mg. P...0·105 %,

a very similar result.

Treatment of dried globulin with alcohol or ether gives a yellow, rather fatty extract, which contains phosphorus. This extract is only separated from globulin with some difficulty. I have tried various means; treatment of the finely powdered globulin in a Soxhlet apparatus with ether; prolonged shaking with alcohol and ether; and boiling with alcohol, after Plimmer. The latter was the most effective; an extract could generally be obtained by its means from samples which had been previously treated in other ways. I found, however, that though most of the extract could be obtained after some eight to ten hours boiling, that further small quantities could be got by more prolonged boiling. In no case could I get anything like a phosphorus-free globulin. I quote two experiments in which the globulin, well powdered, was boiled with successive portions of alcohol for periods of seven hours, until no more extract was obtained.

(1) 0·5265 g. globulin gave ext. cont. 0·3096 mg. P; 0·056 %. Boiled 21 hrs.
(2) 0·68625 g. ,, ,, ,, 0·3207 ,, ; 0·048 %. ,, 35 ,,

Thus about half the original phosphorus is got rid of in the extract. In previous experiments in which prolonged shaking (200 to 300 hrs.) was adopted, not more than 30 or 35 % of the phosphorus was eliminated. I also shook solutions of crude globulin and diluted serum directly with ether. In the former case the results were negative, though in one or two experiments minute traces of an extract similar to that looked for were obtained. With serum, no trace of the extract looked for was found, but an orange pigment was extracted without much difficulty. From pure or nearly pure pseudo-globulin no extract could be obtained by any of these means, though

small quantities were found in samples containing 3 or 4% of globulin, as would be expected. The amount of extract obtained from globulin measured by weight was some 8–10% of the globulin treated.

In view of the difficulty of completely extracting solids by liquids and especially the very slow action of the extracting liquids in this instance, it is tempting to suppose that the whole of the phosphorus in globulin belongs to some lecithin-like body or bodies which would appear closely connected with the globulin, but not part of the molecule. These would then amount to no less than 15–20% by weight of the globulin. On the other hand the present facts do not warrant us in accounting for more than half the phosphorus in this way.

Summary.

1. There are two proteins of ox-serum insoluble in half-saturated solutions of ammonium sulphate, saturated magnesium sulphate, or sodium chloride; the historic water-insoluble globulin, and the water-soluble body pseudo-globulin.

2. These two bodies cannot be split up further by means of fractionation with salt and water.

3. Intermediate fractions are shown to be mixtures of these two bodies.

4. Globulin contains, or is closely associated with phosphorus, rather more than 0·1 mg. P% being found. About half this belongs to a fatty, lecithin-like body which amounts to some 8–10% of the globulin freed from pseudo-globulin. Apparently no part of this body is detached from globulin through prolonged treatment with acids, alkalies, or salts.

5. Pseudo-globulin does not contain phosphorus.

6. Repeated precipitations of globulin at constant volume finally give filtrates in which the amount of protein is constant. Globulin, presumably, can thus be freed from pseudo-globulin.

7. Pseudo-globulin can be freed in a similar way from albumin, the separation being considerably easier.

8. Pseudo-globulin can also be freed, or nearly freed, from globulin by suitable methods of fractional precipitation.

9. The presence of phosphorus has given us an independent test for following the progress of the latter separation. The result has been to establish the validity of the general method of fractional precipitation in this case, to give important indications as to the most suitable method to use, and to estimate the value of the solubility tests.

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L. THE CHEMISTRY OF THE LEUCOCYTOZOON SYPHILIDIS AND OF THE HOST'S PROTECTING CELLS.

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Introduction.

The life-history of the Leucocytozoon Syphilidis has been fully described by McDonagh [1913, 1, 2]. The present communication represents an attempt to elucidate the micro-chemistry of the organism in question.

The first consideration will be given to the physical and chemical properties of the staining reagents used, then to the characters of the syphilitic bodies as evidenced by staining *in vivo*; the same by the staining of fixed specimens, in which the protoplasmic and nuclear portions will be dealt with separately. Reference will then be made to some physical characters of the various cells, and attention given to some special chemical features of the syphilitic bodies and of the host's protecting cells. Finally, the physical and chemical characters of the lecithin-globulin complex will be mentioned.

The physical and chemical properties of the staining reagents used.

In all micro-chemical investigations it is important fully to consider the chemical and physical properties of the staining reagents before drawing any conclusions as to the chemical nature of the structures under consideration. The dyes as a group are almost entirely colloidal in nature, that is to say, they do not readily diffuse through animal membranes. Their colloidal nature is also emphasised by their high molecular weight and absence of crystalline forms. In solution these dyes form typical colloidal suspensions, the size of the particles varying in different cases; consequently we notice that some stains appear quite homogeneous, whilst others show the

well-marked characters of suspensions. Further, many dyes are removed by filtration and may be separated by this means, as, for example, methylene blue occurring in the urine after subcutaneous injection.

Owing to their colloidal nature the small particles of a dye in suspension become electrically charged, and we may for convenience divide up dyes into negatively and positively charged colloids. The negatively charged colloidal particles of a dye will, therefore, show electrical migration to the anode, whilst positively charged colloids will travel towards the kathode. It follows from this also that on mixing a positive dye with a negative dye the charges will be neutralised and the resulting colloidal mixture will then exist in an uncharged condition; *vide* the eosin-methylene blue mixture, the former being negatively charged whilst the latter is positively charged.

But the one great feature of all dyes is that they exhibit the phenomenon of adsorption (Bayliss). By adsorption we mean a "combination" between two substances which is not strictly in the nature of a chemical union, that is to say, in which there is no direct proportionality between the concentration of the solution and the amount adsorbed. There is some kind of physico-chemical affinity between the bodies adsorbed, and those which take them up, but this affinity is more in the nature of a mechanical than a true chemical affinity. To take an example, if a series of solutions of Congo red of varying concentrations be taken, and the same amount of filter paper be placed in each, a part of the dye is taken up, but in relatively larger proportions in the more dilute solutions of the dye.

There are, however, instances where the combination of the dye with the material acted upon is in the nature of a true chemical combination, but these are exceptions rather than the rule, *vide* the staining of nuclei with rongalit white.

A third and most important factor comes into play in the processes of histological preparations, namely, the presence of electrolytes. The dyes as a whole are very sensitive to electrolytes in regard to their adsorption properties and the effect appears to be proportional to the degree of their colloidal nature. Electro-negative dyes, like Congo red, are increased in adsorptive power by the addition of kations, such as lithium, potassium, sodium, ammonium, magnesium and calcium. On the other hand, anions such as hydroxyl, acetate, chloride, oxalate, sulphate and phosphate depress adsorption. With electro-positive dyes, such as toluidine blue, the reverse holds good, namely anions facilitate and kations depress. The effect of electrolytes, however, is much more marked in the case of kations than it is with anions. The salts of the heavy metals which form positively charged

colloidal hydroxides, for example, iron, have a very powerful effect on the adsorption of electro-negative dyes, and in every case the ion promoting adsorption of the dye is carried down with it.

If we turn now to histological preparations, perhaps we may in the light of these observations be able to interpret the changes induced in the act of staining. In the living cell the substance to be stained has a negative charge, as the reaction of the tissues is always on the alkaline side of neutrality, and we know that protein solutions in an alkaline medium are always negatively charged; it follows, therefore, that living cells will take up basic dyes and that electrolytes are not essential for the process. When the cells die the electrolytes attached to the protein constituents of the cell are split off, with the result that the cells now readily take up acid dyes. Further, we know that the fixation of a dye is facilitated by heat, and this fact has been made use of in Altmann's method of acid-fuchsin staining. Mayer has also shown that the Nissl bodies of nerve cells have their affinity for basic dyes destroyed by previous treatment with neutral salts; an observation which further emphasises the importance of electrolytes in the staining act.

Let us now turn our attention to the observations upon the syphilitic parasite and see whether the results obtained can be interpreted in the light of these views of staining. In the first place, we will consider the two principal stains used, viz. pyronin and methyl green. Both these dyes are positively charged colloids and in consequence will be facilitated in their staining by anions, particularly hydroxyl and sulphanion. The pyronin will, therefore, act as a basic dye and this explains why it is precipitated by nigrosine and also diamine green, but not by diazine green. Making use of the chemical characters of the dyes used and reviewing all the results obtained in this light, it is obvious that much more useful and valuable knowledge of the micro-chemistry of the cells under investigation can be ascertained. The specific staining properties of the syphilitic parasite will be again referred to and all we would point out here is the importance of regarding pyronin as a positively charged colloid, and influenced by anions.

The characters of the syphilitic bodies when stained "in vivo."

The simplest and best way to use a reagent for vital staining is to spread a solution on to a slide free from fat and alkali and then allow it to dry in the air. The film should be made just before it is required and not kept several days. The material to be examined is placed upon a cover slip and

the latter so adjusted to the slide that no air remains in between and so that as little as possible of the fluid to be examined exudes at the sides. Examination is possible until the fluid dries, which takes several hours. Ringing the cover slip with wax greatly facilitates the process. The slides used for hanging drop preparations are useless, but a warm stage may sometimes be advantageously employed.

We have used all the stains which have been from time to time advocated, but have not obtained the results which one might be supposed to get from reading the literature about them; therefore, it might be as well to state that neutral red, neutral violet, Bismarck brown, auramine, diazine green, malachite green, tropaeolin 00, and Congo red do not give good vital staining. Owing to the use which is now being made of the azo-dyes in determining the functional activity of certain cells in the body, we attempted several for our method of staining *in vivo*, but by no means with success, because they have such feeble staining properties and are general protoplasmic stains without possessing affinity for certain structures; moreover, they do not possess metachromatic properties. The dyes which gave the best results were aqueous solutions of borax-methylene blue and polychrome-methylene blue; brilliant crystal blue, Nile-blue sulphate and alcoholic solutions of toluidine blue, thionine and azure II. The disadvantage of the alcoholic solutions is that they must be diluted well before use as crystals so readily form, and this is especially the case with thionine. Azure II stains deeply, but unfortunately has no metachromatic properties. The intracellular stages are perhaps better depicted by the alcoholic stains, but taking everything together the results are not so good as when the aqueous solutions of either borax-methylene blue or brilliant crystal blue are used: and, of the last two, the former is superior. The metachromatic properties of borax-methylene blue are dependent upon the basic sodium biborate, which acts upon methylene blue by producing methylene violet which, as a basic dye, shows affinity for acid substances, and methylene red which is an acid dye and so shows affinity for basic substances.

We tried various other bases with methylene blue with the hope of obtaining greater metachromatic action by substituting a base of higher valency for the borax, for which purpose we employed colloidal aluminium hydroxide. The methylene blue remained unaltered, no doubt owing to the fact that the aluminium hydroxide did not contain sufficient free hydroxyl ions and was in itself an unstable colloid and therefore had no action upon the positively charged methylene blue.

Borax-methylene blue, when freshly prepared, has practically no

metachromatic action, but this property increases the longer the stain is kept, until finally the methylene red becomes the stronger dye and stains the cells just as the methylene violet does. Borax-methylene blue appears to be at its best when it has been kept for a year or two. As the methylene red scarcely comes into play in the fresh solutions, no harm is done by adding 0·1 g. eosin to 100 cc. borax-methylene blue, as a true chemical compound results. The eosin picks out the granules in the polymorphonuclear leucocytes, also stains brilliantly the eosinophile granules, but not one of the stages of the syphilitic organism. When we had learnt that the syphilitic organisms contained lecithin in the form of a lecithin-globulin complex, and being aware of the affinity that this complex shows towards dextrose, it struck us that it might be possible to increase the staining properties of the organism by adding dextrose to borax-methylene blue. Although the dextrose did not carry the colloidal dye particles to the cells in question, it nevertheless was taken up by every cell which contained the lecithin-globulin complex, since the protoplasm of such cells swelled and absolutely refused to stain, but, owing to the swelling, were as easily discernible as if they had stained, consequently the plasma cells and syphilitic bodies could be well studied, as their nuclei were not prevented from staining. Witnessing the act of impregnation in a dextrose-borax-methylene blue specimen, we were able to compare it with what we had previously seen. The nucleus of the female appeared to float about, surrounded as it was by the clear ring of unstained protoplasm; when first seen one end of the spirochaeta had already entered, as one extremity was fixed to the nucleus; the spirochaeta was also thickened (due to the dextrose). The one end of the spirochaeta remained fixed to the female nucleus and, in spite of knocking against all the other cells in its progress, it remained attached to the same spot, but did not enter any further. Suddenly the female cell stopped and the spirochaeta pallida vanished inside, but 55 minutes had elapsed before this happened. No further change was noticed in the female cell, as it had not stained very well, but about four minutes later it became very active and discharged a clear non-staining polar body which seemed to be emitted with some force. A few seconds later another clear polar body was extruded and then the female cell came to a standstill again. It is possible that the dextrose made these polar bodies appear bigger than they really were, as each was certainly 2–3 μ in diameter.

From what has been stated it will be easily seen that a description of the syphilitic organism *in vivo* from its reactions will entirely depend upon the characters of the borax-methylene blue which is used, and as impregnated

female cells do not stain with eosin or the methylene red of freshly prepared borax-methylene blue, the increase in the basicity resulting from impregnation cannot be very great. It is far more probable that no change in reaction occurs and that the reason for staining with methylene red, a fact which we have frequently observed, is due to an increase in the reducing action, as will be shown later.

The sporozoites may remain for some time unstained or stain immediately a dense violet. The intracellular phases stain late and the early ones show an affinity for the methylene violet moiety, whilst the late ones, viz. the coils, take up the methylene red. The females before fertilization remain unstained except their chromatic network and blepharoplasts which stain immediately with methylene violet. The spirochaeta pallida stains pink and when it has impregnated a female cell and the whole cell has come to a sudden standstill, a pink diffuse stain comes over the cell like a mantle. The sporozoites while in the spore cysts show a greater affinity for methylene red than methylene violet, and some spore cysts are seen which stain distinctly metachromatically.

In staining *in vivo* with a negatively charged colloid it follows that alkaline basic dyes will react best, and this has been found to be the case; and the reason why neutral red, neutral violet, Bismarck brown, auramine, diazine green, malachite green, tropaeolin 00, and Congo red were found not to give good results is simply the fact that they are negatively charged dyes and therefore cannot stain cells which contain colloids in solution with a negative charge existing in a medium on the alkaline side of neutrality. It follows that good staining can only be obtained by using dyes with a positive charge, hence the reason why borax-methylene blue and polychrome-methylene blue serve so admirably, as it is only under such conditions that adsorption can come into play.

Summary. Basic stains are the most suitable for *in vivo* work, and of these borax-methylene blue is the best. Owing to the presence of a lecithin-globulin envelope the syphilitic bodies can be made to stand out clearer by adding dextrose to the stain. The varied affinity shown by the different bodies on the one hand for methylene violet and on the other hand for methylene red is due to the prevalence of a substance which has strong reducing properties (lecithin-globulin) and not to a change in the reaction.

THE CHARACTERS OF THE SYPHILITIC BODIES WHEN STAINED IN FIXED SPECIMENS.

Both Pappenheim and Martin Heidenhain explained the specific action of methyl green for chromatin as being due to the breaking down of the weak basic salt by the strong nucleic acid radicle; on the other hand the nucleus did not stain with pyronin which they regarded as a stronger basic salt. As a matter of fact methyl green, being a triamino-stain, is by far a stronger base than the diamino-stain pyronin, the basicity of which is also diminished by its O-ring formation. Furthermore, acetic acid increases methyl green staining and if acids combine with the free amino-group of the salt, acetic acid would have done this before the nucleic acid got a chance. Therefore, the explanation of its action, which had held sway for some years, cannot be the correct one.

A stain which had been largely used by Unna, namely rongalit white, was found to resemble methyl green in many respects and was known only to stain the oxygen positions of the tissues.

Rongalit white is the leuco-base of methylene blue which is prepared with sodium sulphite and formalin. It is therefore a colourless and basic mixture and the methylene blue is only brought out as a dye in the presence of oxygen. As rongalit white stains the nuclear part of the cell, Unna concluded that methyl green also picked out the oxygen foci of the tissue and was, therefore, a reduction-sensitive stain.

By a series of experiments Unna showed that methyl green was far more sensitive to reducing substances than methylene blue and that malachite green came in between, but that such reducing agents as grape sugar and hydroxylamine were without effect, i.e. did not decolourise methyl green. Another difference between methyl green and methylene blue and malachite green was that the leuco-bases of the last two could be reconverted into their coloured bases by the addition of hydrogen peroxide, but not so methyl green.

Until Unna enunciated his theory of staining by oxidation and reduction we were under the impression that staining depended upon reaction; in other words, that acid substances stained with basic dyes and were therefore termed basophilic, and basic substances stained with acid dyes and were therefore termed acidophilic. No doubt in part this conception is correct, but there is also no doubt that it was carried very much too far. After all, fixed protoplasm is an amphoteric substance, i.e. it can act as a base or an acid; for instance, the protoplasm of plasma cells stains well with acid

fuchsin, which is an acid dye, or with pyronin, which is a basic dye. Although it stains with both, it does so better with the latter than the former; therefore under ordinary circumstances, it can be stated that protoplasm, using the word in a very general sense, prefers to act as an acid.

From McDonagh's description of the Leucocytozoon Syphilidis and the coloured plates which illustrate his article [1913, 2], it will be seen that by using Pappenheim's stain (a mixture of pyronin and methyl green) the syphilitic bodies stain with pyronin, but differ from all other cells in that the nucleus also apparently stains with pyronin and with a much deeper red than the rest of the cell. Working on the reaction hypothesis, or on the electrolytic theory, one must assume then that the protoplasm and especially that of the nucleus of the syphilitic organism is strongly basophilic and negatively charged, but we have already shown it to be partly acidophilic from our *in vivo* examinations, when we pointed out that certain phases showed an affinity for methylene red. We have then a paradox and a solution to the problem can only be found if we adopt Unna's theory of oxidation and reduction.

Methylene violet, like methyl green, is a reduction-sensitive dye, although not to the same degree, and the reason why certain phases stain with methylene red is not because the protoplasm is acidophilic, but because it has reducing properties, and as methyl green is far more sensitive than methylene violet, every phase stains with pyronin, while only in those in which the reducing action is greatest is the affinity of methylene violet for nucleic acid overcome, with the result that the reducing substance stains with methylene red. In fixed specimens this reducing substance does not stain very readily with acid dyes, should a basic dye be present as well, because if pyronin is supplanted by acid fuchsin, most of the nuclei of the syphilitic organisms stain with methyl green; therefore, this characteristic pyroninophile substance of the syphilitic organisms is a strong reducing agent, is basophilic, and, accordingly, negatively charged according to the electrolytic theory; but the action of its electric charge is overshadowed by its reducing action. Therefore, we have another extremely important factor coming into play in the act of staining.

Seeing how sensitive a stain methyl green is, it at once appears obvious what caution must be taken in choosing the most suitable fixing reagent, and that any fixing reagent which robs the nucleus of its oxygen will naturally prevent staining with methyl green, and will also alter the action of the medium. Fixing tissue for 24 hours in a 1% solution of platinum chloride increases the capacity of the nuclei for methyl green, but it is an

expensive solution and does not give such good results as some other fixing reagents. Mercuric chloride has the disadvantage that the sections may stain unevenly, since it only increases the capacity for methyl green staining in the situation where it remains and diminishes it in those situations where it is reduced by the tissue. If mercuric chloride is employed in an alcoholic solution as a fixing reagent, quite good sections may be obtained with Pappenheim's stain, and the effect is enhanced if a little acetic acid is added to the mixture. Chrome salts render staining with pyronin and methyl green impossible. Osmic acid alone or in conjunction with other acids diminishes the receptivity of protoplasm to most dyes.

Formalin, owing to the formic acid which it so frequently contains as an impurity, not only diminishes the staining properties of protoplasm, but markedly reduces the power of the nuclei to stain with methyl green. Including other fixing reagents which are seldom used and are not available for obtaining satisfactory sections with Pappenheim's stain, we are left only with alcohol, and from several experiments we have undertaken we are firmly of the opinion that alcohol is far and away the finest fixing reagent we possess at present, as it allows staining with most of the stains in general use, it fixes by coagulation, and forms no chemical compound with the cells and is, therefore, extremely well adapted for the purpose of micro-chemical research when fresh sections are not employed.

We either employ absolute alcohol or 50% alcohol; the former causes shrinking of the intercellular tissue but not so much of the individual cells and its main advantage is that coagulation is immediate. The moment the tissue is removed from the body it is placed at once into absolute alcohol and allowed to rest on wool—whenever alcohol is used a pad of wool should rest on the bottom of the bottle so as to allow the alcohol to remain the same strength throughout while the water extracted from the tissue sinks through the wool; this simple device means also a great saving of alcohol. The tissue remains for 12 hours in absolute alcohol and can then be changed into two further lots of absolute alcohol for 12 hours each or be put back to 50% and gradually taken up in the usual way. Cedar wood oil is used for clearing, as it does not harden the tissues to the same extent as xylene, and before it is put into wax two changes of xylene are used for 1–2 hours each, as wax penetrates better after the tissue has been through xylene. The wax used is a mixture of:

Paraffin (melting point 60° C.) ...	8½ parts.
Stearin	1 part.
Wax	½ part.

The melting point of the prepared article is 53° C. and the tissue is left in this, changed three times, for about six hours altogether. The whole of the secret of getting good paraffin sections is to be absolutely certain that the tissue is fully dehydrated.

The tissues can also be fixed in 50% alcohol, allowed to remain in the solution for 24 hours and then for 24 hours each in 70% and 90% and 12 hours each in three changes of absolute alcohol, and so on as before. There is not so much shrinkage of the tissue, and most excellent Pappenheim-stained sections may be obtained; but, owing to the solubility of certain substances in 50% alcohol, this method cannot take the place of absolute alcohol in micro-chemical research.

For some tests of minor importance celloidin sections are preferable, but for general purposes we much prefer paraffin, as thinner sections can be obtained; they can be more easily fixed to the cover slips, and one does not have to go through the troublesome procedure of removing the celloidin, which is necessary when aniline dyes are being used, owing to the intense avidity which celloidin has for many of them.

If Pappenheim's stain is to be used we proceed as follows: Roughly two parts of a saturated aqueous solution of pyronin are mixed with one part of a saturated aqueous solution of methyl green immediately before use, or until the mixture assumes a red-purple colour. In this stain the sections may be left from 5 minutes indefinitely as overstaining is impossible. After being in the stain the sections are transferred to a freshly prepared distilled water solution of resorcinol, which is just used for washing off the stain, i.e. about a minute, and then they are put into a freshly prepared absolute alcoholic solution of resorcinol and kept in until all the superfluous stain has come away.

These resorcinol solutions are absolutely essential, as they act as mordants and we regard mordanting after staining as superior to Unna's method, which consists in adding carbolic acid to the stain, which enables the stain to be prepared and always ready for use. The stain is sold under the name carbol pyronin methyl green. The great disadvantage of the ready prepared stain is that the pyronin comes out too quickly in the dehydrating process, while if resorcinol is used this is not the case. The amount of resorcinol crystals used in the first watch glass is about 0·3 g. and in the absolute alcohol watch glass, just double the quantity. From the resorcinol the sections go through three changes of absolute alcohol, two of xylene, and are then mounted in balsam.

As ethyl alcohol may abstract the pyronin stain from the sections, clearing fluid may be used to take its place, such as chloroform, lavender oil or

bergamot oil. Clove oil should never be employed owing to its powerful reducing action.

Xylene fortunately acts indifferently, but Canada balsam in time, owing to its reducing action and being an acid, destroys the staining effect; dammar dissolved in xylene would form a better medium for preserving the section. For a year or two, or even longer, sections stained in the above method and mounted in Canada balsam show a much sharper contrast of colour; the pyronin stands out clearer, the orange colour of the mast cells is more distinct, but the methyl green staining is somewhat weaker, and as time proceeds it is the methyl green stain which first disappears. Some sections prepared seven years ago are as good as and in many respects, owing to increase of sharpness, better now than then.

In a Pappenheim-stained section the protoplasm of the groundwork and connective tissue cells stains a rose pink and has a finely granular appearance, whereas the protoplasm of the plasma cells stains a clear red. All nuclei stain green, the nucleoli a brilliant red, the mast cell granules orange and all bacterial and protozoal bodies red. The protoplasm of the syphilitic bodies stains a rose pink to red, and the nuclei a deeper red. The difference in the rose pink to red of the protoplasm being most marked in the female cells is dependent upon whether they have been impregnated or not, as the fertilised female cells and zygotes always stain deeper. At first sight one might conclude that the supposed nuclear part of the syphilitic organism stains deeply with pyronin, because it contains no nucleic acid; but we have endeavoured to prove that such a surmise is incorrect.

We added acetic acid to the alcohol in which the tissues were fixed in the proportion of 1 cc. glacial acetic acid to 75 cc. either absolute or 50% alcohol, with the hope that if any nucleic acid was present the acetic acid would precipitate it. When the sections were stained we found that the general pyronin staining had not been interfered with, that the methyl green staining was strongly intensified, and that some of the nuclei of the syphilitic bodies stained a brilliant green, proving at once that they contained nucleic acid. The addition of acetic acid in the alcohol used for fixing, for ordinary staining with pyronin and methyl green gives better results than when alcohol is used alone, owing to the fact that apart from the nucleic acid being precipitated, the swelling action of the acetic acid on the cells is counterbalanced by the shrinking action of the alcohol and *vice versa*. For special staining, as when the demonstration of micro-organisms is required, alcohol alone is the one and only fixing reagent.

As methyl green is one of the ingredients of Ehrlich's triacid stain and

the red dye, acid fuchsin, is an acid dye in contradistinction to pyronin which is basic, we stained some sections with this mixture, with the result that the protoplasm of the syphilitic bodies stained red, while the nuclei stained green, another proof that the nuclei contain nucleic acid. We can, therefore, assume that there is some substance either in or over the nucleus of the syphilitic parasites which is a strong reducing agent, as it prevents the methyl green from getting at the nucleus and that it prefers basic to acid dyes.

Our next step was to try to determine the reducing action of this substance and also its degree of solubility in various reagents.

Reducing action. (*a*) Sections were stained for 1–2 minutes in a freshly prepared 1 % solution of potassium permanganate, then washed in water, decolourised in oxalic acid if overstained, dehydrated and mounted in balsam. All protoplasm has a reducing action and consequently stains brown with potassium permanganate while the nuclei remain unstained. The protoplasm of the syphilitic bodies has a greater reducing action than ordinary granoplasma, but the nuclei do not stain and can be counterstained with methyl green; therefore, the reducing body has not a very marked action on potassium permanganate.

(*b*) Sections were placed for 5 minutes in a mixture of equal parts of a 1 % solution of ferric chloride and a 1 % solution of potassium ferricyanide. It is imperative that these two solutions shall only be mixed immediately before use, as if allowed to stand a blue precipitate is slowly formed. The reducing action of the granoplasma converts the ferricyanide into ferrocyanide with the result that where the reduction is greatest a beautiful Berlin blue colour is formed in the presence of the ferric chloride. Ordinary granoplasma has a weak reducing action and so stains green; the granoplasma of plasma cells and the syphilitic bodies have a stronger reducing action and so stain darker, while the nuclei of the ordinary cells do not stain, except some of the "nuclei" of the syphilitic bodies, which stain a faint Berlin blue colour. Red blood corpuscles also give the Berlin blue reaction and so do the aminoplasma cells, both to a more marked degree than the nuclei of the syphilitic bodies. Ordinary nucleoli have likewise a reducing action on ferric ferricyanide.

There is something quite peculiar in the appearance of the syphilitic bodies stained with potassium permanganate and ferric ferricyanide, because in the former, when counterstained with methyl green, the nucleus appears smaller than it really is; there is less to take the methyl green. In both it appears irregular, and scattered here and there about the nuclei are small non-staining transparent areas.

We will deviate somewhat from our course here and dwell upon the aminoplasma cells.

The aminoplasma cell is a form of plasma cell which Unna has called, from his examinations thereof in fixed specimens, Hyaline plasma cell. The term "Hyaline" rather suggests some relationship to cartilage, although it is very largely used for substances of which the observer has no knowledge. Now, hyaline cartilage is a strongly basophilic substance owing to its chondroitin-sulphuric acid radicle and therefore possesses great affinity for methyl green. Unna's hyaline plasma cells are, on the other hand, acidophilic and contain no acid radicle; furthermore, they have very strong reducing properties and so cannot stain with methyl green and, as we shall show presently that this reducing action is due to tyrosine, we consider that the best name for them is aminoplasma cells.

The cell is frequently to be met with in syphilitic material, but it is also to be found in any very chronic inflammatory lesion, viz. Rhinoscleroma and Ulcus Molle Serpiginosum. In *in vivo* specimens an aminoplasma cell is apt to be mistaken for a zygote, owing to the affinity which both have for methylene red, but the distinction becomes clear when it is borne in mind that the former may vary in size from 7–14 μ or more in diameter, that it may have no nucleus, that the nucleus stains homogeneously with methylene violet, that it may be situated in the centre of the cell or at the periphery and that it sometimes possesses the power of motion and may be extruded and finally excluded from the cell altogether. In the aminoplasma cells also are generally to be seen dots, masses or strands situated anywhere and irregularly scattered about the cell, which have no connection with the nucleus, although they stain deeply with methylene violet.

In fixed specimens the appearance of these cells is very different and instead of being round, homogeneous cells, they are often irregular in shape and divided up into irregular sized loculi or balls of protoplasm, many of which become loose and scattered about in the tissue. These balls stain with safranin, and acid fuchsin, and give a Berlin blue reaction with ferric ferricyanide. They do not stain well with pyronin, but in some specimens strands of protoplasm are to be noticed in between the loculi which do stain with pyronin. The strands are no doubt the same as the dots, masses and strands which were described in the *in vivo* method as showing an affinity for methylene violet.

These ballooned plasma cells have in some cases lost their nuclei, whilst in others the nucleus is lengthened out and fits one apex of the cell like a cap does the head, and not infrequently sends stringlike processes down over

the cell protoplasm. These cells are no doubt degenerated cells, because the protoplasm gives amino-acid reactions and in the most degenerated cells the nucleus gives the histone reaction and fails to stain with methyl green.

From what has been said it will at once be seen that the syphilitic bodies bear points of resemblance to the aminoplasma cells, but that they differ in the very striking point that the most reducing part of the syphilitic body stains deeply with pyronin, whilst the most reducing part of the aminoplasma cell stains faintly with pyronin, and, as we have shown that the reducing substance of the former is basophilic, it at once appears obvious that that of the latter is more acidophilic.

The Berlin blue formation is a fixed chemical process between tissue and reagent, since, although the colour can be caused to vanish with alkalies, it immediately returns on the addition of an acid, and, as the feeble reduction areas are more quickly decolourised than the firm Berlin blue areas, weak alkalies may be used to decolourise the former and the protoplasm of the cells can then be counterstained with an aniline dye.

(*c*) The third reaction we tried was with tetranitrochrysophanic acid ($C_{15}H_6O_4(NO_2)_4$). It is a crystalline product obtained from chrysarobin which is dissolved in acetic acid and treated with nitric acid. The reagent is insoluble in water, and owing to the reducing power of ethyl and methyl alcohol it has to be dissolved and kept in chloroform or xylene.

After staining for 10 minutes the sections are returned to chloroform, put through three changes and through xylene, and then mounted in balsam. Weak reducing agents stain a pale rose red, strong reducing agents stain red. The protoplasm stains pale rose red, nuclei remain unstained, syphilitic bodies stain a deeper red, but the contrast is not so clear as in (*a*) and (*b*).

In tissues there are four chief classes of bodies:

1. Proteins. 2. Carbohydrates. 3. Fats.
4. Cholesterol, lecithin and allied lipoids.

All four groups possess reducing properties in varying degrees, but the second we can rule out in the present discussion as will be shown later. Therefore, the reducing substance must be a protein, a derivate of a protein, a fat, or a lipoid. Speaking generally, pure proteins, fats (olein excepted) and lipoids are not strong reducers, but derivates of proteins are, especially the amino-acids, and here is appended a list of amino-acids with their action on potassium permanganate and the ferric ferricyanide mixture (after Unna).

Amino-acids	$KMnO_4$	Iron mixture
Asparagine	–	–
Alanine	–	–
Phenylalanine	–	–
Leucine	+	+
Glutaminic acid ...	+	+
Glycokoll	+ +	–
Cystine...	+ +	–
Tyrosine and Tryptophane	+ + +	+ + +

The amino-acids *par excellence* which give the Berlin blue reaction are tyrosine and tryptophane, and to prove that these plasma cells contained tyrosine we stained some sections in Millon's reagent and succeeded in getting the recognised reaction. Millon's reaction was, on the other hand, very feebly marked in the syphilitic bodies; therefore, the reducing substance of the syphilitic bodies is not dependent upon tyrosine for its property.

So far as amino-acids are concerned we can say that the syphilitic bodies contain none free. Feeling that the protein molecule existed as such, we first directed our attention to the albumoses and repeated all Unna's experiments.

(*a*) *The protoplasmic portion of the syphilitic organism.*

The arbitrary division of albumoses is, in our opinion, not quite justifiable, and to say that granoplasma is a very special albumose, as Unna attempts to do, can scarcely be correct, since the granoplasma of connective tissue cells behaves differently from that of plasma cells; the greatest difference is also to be noticed in the individual plasma cells themselves depending upon their stage of development and degeneration, and lastly that of organisms and protozoa is as different again, and all behave differently according to the method of fixation.

Unna states that granoplasma is a deuteroalbumose and not a primary albumose. Although it differs from the former owing to its greater insolubility and in this respect resembles an acroalbumose which belongs to the latter group, the assumption is made that granoplasma is a deuteroalbumose, which has probably been formed from an acroalbumose.

To make this very complicated part of our work as clear as possible, it may be said that the most soluble granoplasma is that met with in the connective tissue cells and groundwork, then comes the granoplasma of some plasma cells, then of other plasma cells, then of the embryonic lymphocytes and nucleoli, and finally of the syphilitic bodies; but here we must halt for a moment, as in the syphilitic bodies we are dealing with two distinct proteins, one rather granular, which stains lighter with pyronin, the other

highly refractile, which stains deeply with pyronin; the former is the groundwork or granoplasma of the cell and resembles ordinary granoplasma; the latter may cover the whole cell or only the nucleus and is extremely resistant to reagents, and therefore does not resemble ordinary granoplasma; this is the protein which we will call the pyroninophile substance.

As the granoplasma of the syphilitic bodies resembles ordinary granoplasma, the protein of the syphilitic bodies will be referred to as the pyroninophile substance, since it is the chemistry of this substance that we wish to unravel.

Unless otherwise stated, the following experiments were undertaken with sections which had been fixed in 50 % alcohol and which were placed in the different reagents for 12 hours at room temperature and then stained with pyronin and methyl green.

1. In distilled water granoplasma begins to dissolve, the action is very much quicker at 37°, but the syphilitic bodies remain unaltered. If kept in for several days, the avidity for pyronin disappears and the nucleic acid is left behind to stain with methyl green. One may say that the protein of the syphilitic bodies is insoluble in water; this rules out protoalbumose and deuteroalbumose. If normal saline is substituted for distilled water, the action is much the same and the syphilitic bodies are still insoluble; by this test acroalbumose is excluded.

2. 30 % alcohol behaves like normal saline and dissolves a greater portion of the granoplasma, but has no action on the syphilitic bodies. In 60 %, 70 %, 80 %, 96 %, and absolute alcohol the granoplasma remains mostly intact, depending upon the concentration, as no granoplasma is soluble in absolute alcohol. In no percentage of alcohol are the parasites dissolved; this excludes at once peptone and protoalbumose, also deuteroalbumose α, which is soluble in 70 % alcohol and all the other deuteroalbumoses which are soluble in 80 % alcohol and upwards, except thiodeuteroalbumose and glycodeuteroalbumose β II, both of which are insoluble in absolute alcohol.

3. In a 10 % solution of metaphosphoric acid granoplasma and the syphilitic bodies are insoluble, owing no doubt to the precipitation of all proteins by the acid; this is likewise the case with 1 % phosphomolybdic acid, alone, or with 1 % hydrochloric, 1 % phosphotungstic acid, picric acid, and weak solutions of the mineral acids. These tests rule out protoalbumose, which is soluble in picric acid and dilute mineral acids, and Kühne's heteroalbumose which is also soluble in dilute mineral acids. It is very difficult to work with the above acids owing to the fact that they all prevent staining with methyl green, and everything stains a diffuse red with pyronin.

4. Granoplasma and the proteins of the syphilitic bodies are insoluble in all strengths of acetic acid, and the reason why the nuclei stain green, giving the first impression that the pyroninophile substance over or in it has been dissolved, is only due to the marked precipitating action of acetic acid on nucleic acid.

5. Granoplasma is very soluble in a 1 or 2 % solution of boric acid but insoluble in a 5 % solution; the syphilitic bodies, on the other hand, retain their affinity for pyronin. Such a pretty instructive picture is obtained by leaving a section in 1 % boric acid for 12–20 hours at ordinary temperature and then staining in the usual way with pyronin and methyl green, that more than a passing mention is desirable. The granoplasma of all the cells has dissolved, the nucleoli have vanished, those embryo lymphocytes which stain red and might be confounded with certain phases of the syphilitic organism now all stain a brilliant green, and the only bodies which stand out a brilliant red colour are the syphilitic parasites. So in this very simple method we have as fine a differential stain as the Ziehl Niellsen for tubercle bacilli.

6. In 1 % potassium ferrocyanide not only does the ordinary granoplasma disappear, but the protein of the syphilitic bodies does also, with the result that only the nuclei stain with methyl green. If acetic acid is added to the potassium ferrocyanide the granoplasma and protein of the syphilitic bodies remain unaltered and stain in the ordinary way.

7. In a 2 % solution of copper sulphate the granoplasma has gone, nuclei remain, nucleoli have disappeared, also the granoplasma of the aminoplasma cells, the groundwork protoplasm of the syphilitic female bodies has vanished, but the pyroninophile substance over the nucleus remains intact and stains with pyronin, and most of the spore cysts stain red.

8. In 1 % caustic potash nuclei and all have dissolved.

9. In mercuric chloride and alcohol there is no change.

10. The syphilitic bodies remain unchanged after treatment with a 1–10 % solution of lead acetate; nucleoli are likewise not dissolved in this reagent.

From these experiments it is clear that the pyroninophile protein of the syphilitic parasites is not ordinary granoplasma, it is not an albumin, albumose or peptone; this leaves us with only globulin. So when the insolubility of the protein under question is considered, we think we are justified in saying that it is a globulin, or, as we shall see later, a globulin complex. If the sections have been fixed in absolute alcohol which prevents the extraction of salts and acts as a very powerful coagulant, many of the

above-mentioned substances fail to make any alteration, boric acid, for instance, being innocuous, and the syphilitic protein does not dissolve in potassium ferrocyanide. To produce the same results sections must be left in the reagents for several days.

50 % alcohol can extract electrolytes from the cells, hence they become less negatively or positively charged and the charge may be still further diminished by reagents, and as electrolytes are essential for the staining of fixed specimens, their complete abstraction will result in the pyronin not staining. Hence, it may be quite wrong to say that this or that protein dissolves in this or that solution, as it may be only its electrolytes which are removed; therefore, as previously stated, the arbitrary division of the cell proteins is not justifiable.

Absolute alcohol extracts few, if any, of the electrolytes, hence staining is not interfered with. As the syphilitic protein when fixed in 50 % alcohol resists reagents so remarkably, it can from what has just been said be assumed that the salts or electrolytes are firmly bound up with the protein. This would not be the case unless the protein was itself also bound up in a highly organised and stable complex, so we have the first clue in this simple observation that the pyroninophile substance of the syphilitic parasite is a protein (globulin) complex.

(*b*) *The nuclear portion of the syphilitic organism.*

After treatment with acetic acid before staining with pyronin and methyl green, or employing Ehrlich's triacid mixture, in order to get the nuclei of the syphilitic bodies to stain with methyl green, on careful examination marked differences can be discerned between the parasitic nuclei and those of other cells. In the former the methyl green stain gives a purer green colour, the stain is more brilliant and it is evenly distributed throughout the nucleus, or in other words, is homogeneous. If small lymphocytes be now examined, or the nuclei of plasma cells, and contrasted with the above, it will be noticed that the green is darker and has a mixture of blue or black; it is duller and moreover distributed into dots and strands, which are the chromatin bodies and filaments. If attention is now paid to the dividing cells and the embryo lymphocytes it will be noticed that the green resembles that met with in the syphilitic bodies and that the stain is again homogeneous. The only phase of the syphilitic organism which at all resembles in colour the lymphocytes or nuclei of the plasma cells is the spore cyst, or rather the sporozoites which

the cyst contains. This very simple observation is yet another very important argument in favour of McDonagh's view, since the nuclei of the developing syphilitic bodies resemble those of the developing lymphocytes and the dividing plasma cells, while the sporozoites which have no further need to develop and are in the resting stage resemble the mature lymphocytes and resting plasma cells. Degenerated nuclei behave quite differently; the division into chromatic filaments becomes more marked, the methyl green stains them bluish, then not all and at this stage the slender chromatic filaments stain red with the pyronin, or a diffuse red stain of the remaining protein may result; therefore, the syphilitic bodies are not degenerated nuclei.

The chemical substance of the nucleus is a compound of a protein and nucleic acid and is therefore called a nucleoprotein. This nucleoprotein on hydrolysis breaks down into protein and nuclein; the nuclein into protein and nucleic acid; the nucleic acid into purine bases, viz. guanine, adenine, xanthine and hypoxanthine, pyrimidine bases, viz. thymine, cytosine and uracil, pentoses and phosphoric acid.

Methyl green only stains nuclein and nucleic acid, whilst the protein stains with pyronin; therefore the reason why degenerated nuclei stain in some cases with pyronin, is that the nucleic acid has become further split up, while the protein remains behind.

(c) *Action of reagents on nuclei.*

We next tried a series of experiments by leaving sections which had been fixed in 50% alcohol for 20–24 hours at room temperature in several reagents to see if we could get any different reactions with the nucleic acid of the syphilitic bodies, and that of ordinary cells. The sections were stained with pyronin and methyl green, and every experiment which was undertaken was also repeated with sections of the soft roe of a herring (*Clupea harengus*).

1. Leaving a section of roe for 20 hours in a concentrated solution of ammonia results in a partial disappearance of the nucleic acid, but the cells still stain with methyl green and retain their form; the chief difference from the normal is the appearance of a diffuse mass of protein which stains red with pyronin and is no doubt a mixture of histone and protamine.

In the syphilitic section the nucleoprotein has also been broken up; in some nuclei the nucleic acid has disappeared altogether, in other nuclei there are masses of it left as the red field is dotted here and there with some blue masses (methyl green). The granoplasma of the cells has dissolved. The

nuclei of the syphilitic bodies have partly gone and the granoplasma has completely disappeared and also the pyroninophile substance.

Nucleoli have mostly gone, and the aminoplasma cells completely. The best maintained of the syphilitic bodies are the sporozoites in the spore cysts which still stain quite intensely with methyl green and are, on the whole, even less damaged than the nuclei in the herring's roe. Therefore, the sporozoites are not only extremely rich in nucleic acid but also extraordinarily resistant to chemical reagents.

2. After leaving sections of roe in saturated sodium chloride solution all the nucleic acid disappears and all that is seen is a diffuse mass of histone which stains well with pyronin.

In the syphilitic sections the granoplasma of the cells is well preserved, if anything the pyronin staining of the protoplasm of the plasma cells is increased. The nuclei on the other hand are very much altered; they stain homogeneously a slate-grey colour; the chromatic bodies and filaments are not to be seen, but the nucleic acid is less disturbed than in that of the fishes' roe. The nucleus of the syphilitic parasite does not stain with methyl green, not because the nucleic acid is dissolved but because the brilliant refractile pyroninophile substance has been precipitated and therefore has had its properties intensified.

The nucleoli are well preserved and the aminoplasma cells are intact.

3. A section of roe which has been treated with a 1 in 3 solution of magnesium sulphate has lost all its nucleic acid, no cell outline is even discernible and all that remains is the precipitated histone, which stains especially brilliantly with pyronin.

In the syphilitic sections the granoplasma has partly dissolved, the nuclei stand out, stain a brilliant green and are intensely refractile, looking like pieces of green glass. The great difference between the nuclei found in the roe and in the syphilitic section can possibly be explained by the fact that in the latter the nucleic acid has not been extracted before the precipitation of the histone, with the result that the nucleoprotein is maintained, as is the case in sections which have remained in potassium ferrocyanide. All nucleoli have vanished. The nuclei of the syphilitic phases stain a brilliant green and are much better preserved than the nuclei of other cells; all the pyroninophile substance has completely disappeared.

4. The only difference noticed in a section of roe which has been in a 0·1 % solution of calcium chloride is that a trace of histone has been abstracted from the nuclei.

In the syphilitic sections the staining is not quite as good as under

ordinary circumstances. The pyroninophile substance is not destroyed, but the sporoblasts and sporozoites show a greater affinity for methyl green than usual; therefore a trace of the body is soluble in calcium chloride. These sections show up some points which we have frequently observed in other sections, and which have more than once raised the question as to whether we were dealing with spore cysts or the degenerated nuclei of plasma cells.

In some spore cysts which stain with methyl green, bright red bodies are to be seen resembling nucleoli. There may be one or more, varying in size, and frequently the largest red mass lies on and looks as if it was part of the biggest spore body. In this latter observation lies the solution. This large spore body is a part of a sporoblast which has to divide still further to form sporozoites and, as mentioned above, the oldest sporozoites have generally lost their pyroninophile constituent, which would not be the case with undeveloped sporozoites. The pyroninophile substance is no doubt separated off and breaks up into fragments in the groundwork of the spore cyst.

When the nucleus of a plasma cell degenerates it does not break up into a mass of circular bodies, but into a ring of bodies, some of which are circular and others oval; and there is nothing in the centre except a few strands which stain red with pyronin.

5. In 2·5 % sodium chloride all sections show the points brought out by the preceding reagent in a slightly more pronounced degree.

6. 20 % ammonium sulphate solution has the effect of so breaking down the nucleoprotein of the nuclei found in a section of roe that no distinct nuclei are seen, only a diffuse red purple colouration of the nucleic acid being left upon a deeply red diffuse groundwork of histone.

In syphilitic sections destruction is not nearly so marked, so far as the *toute ensemble* is concerned; the pyronin stain seems to have been increased probably owing to some abstraction of histone from the nuclei, as the staining capacity of the nuclei is very much weakened. The pyroninophile substance is maintained.

If specimens fixed with absolute alcohol are used, quite different results are again obtained. Nuclei are not affected by concentrated ammonia, but the pyroninophile substance dissolves. In 20 % NaCl nucleoprotein has been split up and part of the nucleic acid dissolved, but the pyroninophile substance is unaltered. If the syphilitic bodies are closely examined, a clear white halo surrounds the nucleus and tiny areas of white are to be seen in the nucleus. The refractility of the protoplasm of the plasma cells is markedly diminished while that of the syphilitic bodies appears to be increased, which has the effect of strongly differentiating them from the other cells.

7. In 0·6 % lithium carbonate the granoplasma of cells has mostly disappeared, the nuclei stain homogeneously and of a bluish colour; nucleoli are well maintained, and the syphilitic bodies remain practically unaltered. So here again is a fine differential method.

(*d*) *Action of sera on cells.*

We thought it possible that something might be learnt by treating sections with different sera, so the following experiments were undertaken.

Several cc. of blood were withdrawn from a non-syphilitic, a case of early secondary syphilis, and a case of late tertiary syphilis. Great care was taken to have the serum absolutely free from haemoglobin, and when put into the watch glass, a covering of pure toluene was used to prevent any bacterial action. Unless the latter precaution is taken bacteria multiply in the sera and exert a pronounced hydrolytic action on the sections so that even after 20 hours the individual cells are only just discernible. All sera have the same action as normal saline. If specimens fixed with absolute alcohol are treated in the same way, there is no change and in neither case can any difference be detected between the action of the three sera.

Whether the pyroninophile protein of the syphilitic bodies is part and parcel of the nucleus or only its sheath is at first sight difficult to ascertain, but we incline to the latter view for the following reasons.

Morphologically it looks more like a cover, this being especially noticeable in potassium permanganate specimens which have been counterstained with methyl green, as the nucleus stains faintly and appears hazy and gives exactly the impression of being covered with a veil. It is soluble in potassium ferrocyanide and when sections are left for 12 hours in a 1 % solution the nuclei stain better than ever and appear clearer with methyl green, which would scarcely be the case if it was part of the nucleus. Moreover, it gives a faint Berlin blue reaction, which no nucleus is known to do. Further, by staining with dextrose-borax-methylene blue, during impregnation, there appears to be a marked differentiation of ecto- and endoplasm and even the spirochaetae are swollen. If it is not part of the nucleus, in what combination with protein does the nuclein and nucleic acid of the syphilitic bodies exist? That a protein does exist is quite clear, since the nuclei of the syphilitic parasites can be made to stain with acid dyes, viz. diazine green, which mixes very well with pyronin; and moreover, will stain well with pyronin when the nucleic acid has been separated off, when it is found to behave like ordinary granoplasma. In this respect the protein

radicle of the syphilitic nucleoprotein does not differ from that of ordinary cells.

The protein of nucleoprotein is always regarded as quite a special protein, and said to possess strong basic properties and to be extremely rich in hexone bases, viz. lysine, arginine and histidine.

Nucleoprotein is a complex in which the properties of the protein can be very materially altered by the prosthetic group, since we are by no means aware what all the other constituents are and therefore cannot tell when they have been removed; we can never be sure that we are dealing with the protein only.

We have already shown that the division of the proteins of the cell protoplasm is purely arbitrary and the separation of the nucleoproteins is likewise no doubt artificial, since when the protein of the nucleoprotein is separated out it gives the same micro-chemical tests and behaves in the same way to stains as does the cell granoplasma; therefore the syphilitic nucleus does not differ in gross details from the nuclei of its host's cells.

For lysine and arginine we have as yet been unable to find a specific microchemical test, as immersing sections first into a freshly prepared solution of diazobenzene-sulphonic acid and then washing with a 1% solution of NaOH, did not give the characteristic pink colour which is seen in the microchemical test for arginine. Reversing the solutions by using the alkali first made no difference. Whichever method was employed a beautiful stable orange colour, which stained both the protoplasm and the nuclei, resulted.

The well-known test for histidine, namely, bromine water and acetic acid, also gave negative results, both before and after hydrolysing with a 0·5% solution of hydrochloric acid. Oddly enough a section which had been treated with bromine water and acetic acid quite failed to give the orange colour with diazobenzene-sulphonic acid and sodium hydroxide, which looks as if either the histone or the hexone bases had formed a halogen compound, which of course they are well known to do, and the bromo-histidine compound, for instance, does not give the characteristic reactions of the base.

Failing to get the typical hexone reactions we did not expect nor did we succeed in getting a positive imidazole reaction by treating sections with ammonia and silver nitrate. We also tried, but once more with negative results, to get a positive biuret reaction which is obtained with histone; the failure was due to the destruction of the tissues by the strong soda solution, which is unfortunately necessary for the reaction.

All nuclei are stated to contain phosphorus, which is intimately bound up with the nucleic acid radicle of the nucleoprotein and disappears when the

nucleic acid is hydrolysed, and as it is the nucleic acid radicle which shows the affinity for methyl green it is possible that this acid plays a part in the selective action of nuclei for methyl green. The nuclei of the syphilitic parasites, when the pyroninophile membrane covering them is removed or prevented from staining, stain not only brilliantly with methyl green but also show a greater resistance to hydrolytic agents than do the nuclei of ordinary cells; hence it might be expected that the parasitic nuclei were especially rich in phosphorus.

To see whether this surmise was correct, the following experiments were undertaken:

Phosphorus. Both fresh sections and specimens, fixed with absolute alcohol, the latter giving quite as good results as the former, were placed in a mixture of molybdic acid, ammonia and nitric acid, and kept therein at 37° for 10 minutes to 48 hours. One is supposed to regard as inorganic phosphorus that which makes its appearance in the first 10 minutes, but when sections are examined so early only negative results are obtained. After 24 hours good staining effects can be obtained, but the staining is sharper if the sections are left in the mixture for even another day.

The sections are washed well in distilled water and are then placed in a 2% solution of phenylhydrazine hydrochloride, taken direct through alcohol, and mounted in balsam.

The presence of phosphorus is indicated by a green colour, and when the sections are examined it is found that both the protoplasm and the nuclei of nearly all cells are stained. The staining is deepest in the syphilitic bodies and then in the plasma cells; there appears to be no great difference in the staining properties of the nucleus compared with the cell protoplasm, which indicates that the phosphorus in a cell is not restricted to the nucleus.

As nuclei also contain iron the following tests were undertaken:

Iron. A. Inorganic.
B. Organic.

(A) *Inorganic.* Specimens fixed with absolute alcohol were after removal of wax transferred direct from absolute alcohol into a freshly prepared solution of equal parts of 0·5% hydrochloric acid and 1% potassium ferrocyanide, and allowed to remain therein for one hour. By this means the cells did not give the Berlin blue reaction, nor did they even stain green.

Instead of the hydrochloric acid potassium ferrocyanide mixture, a 0·5% haematoxylin solution was employed, which also failed to prove the presence of inorganic iron.

(B) *Organic.* Sections prepared as above were placed in a mixture

of sulphuric acid (4 vols.) and absolute alcohol (100 vols.) and left therein at 37° for 24–48 hours.

After 24 hours the sections were washed in absolute alcohol and some were transferred for half an hour into the hydrochloric acid potassium ferrocyanide mixture, whilst others were stained in 0·5 % aqueous solution of haematoxylin. From both solutions the sections were taken through alcohol, etc. and mounted in balsam. When the former were examined it was seen that the protoplasm of the cells remained unstained while the nuclei stained green, the arrangement of the chromatin remaining unaltered. Other nuclei stained a light Berlin blue and the colour was homogeneous; examined for action on polarised light they were found to react slightly, while one of the other cells showed a trace of reaction. These cells were no doubt the syphilitic parasites; the nuclei of which contained more organic iron than those of the leucocytes and connective tissue cells.

The same difference in degree of staining was also noticeable in the haematoxylin specimens.

Summary. The protoplasm of the syphilitic bodies is strongly pyroninophile, which proves it to possess reducing properties. The reducing action is not so strong as that of the aminoplasma cells and therefore is not due to an amino-acid; and moreover it prefers basic to acid dyes which still further distinguishes it.

The protoplasm is very resistant to reagents and in all respects resembles a globulin. The nucleus in its behaviour to dyes and reagents most closely simulates the nuclei of dividing cells.

Hence, neither can the syphilitic bodies be taken for cell degenerations nor nuclear degenerations.

Further points concerning the reducing action of cells, with special reference to their physical characters.

None of the proteins we have mentioned have sufficient reducing power to form Berlin blue from the ferric ferricyanide reagent; therefore, the reducing action of some of the syphilitic bodies, red blood corpuscles, etc., must be due to a carbohydrate, a fat or a lipoid.

Carbohydrates. Our endeavours to find a carbohydrate in the syphilitic bodies failed and the tests used are only of interest from the negative information derived therefrom.

Keeping sections for some time in a hot solution of Fehling's reagent gave no yellow precipitate. Leaving sections for 48 hours at 37° in a solution

of 0·5 % potassium hydroxide in 90 % alcohol and then immersing them into a 2·5 % solution of dimethylparaminobenzaldehyde in 1 % HCl gave no carmine reaction, which indicates the absence of glucosamine.

Treating sections with a 15 % alcoholic solution of α-naphthol and then examining them in sulphuric acid for the furfurol reaction, owing to the destruction of the tissue, gave no information.

The principal organic tests and group reactions either necessitate the use of strong acids or alkalies; with the former the tissue is as often as not dissolved *in toto*; with the latter it is almost certain to leave the cover slip, swell and become practically unmanageable. Ammonia is the least offender in this way, but unfortunately it cannot as a rule take the place of the potassium or sodium hydroxides.

We tried also leaving fresh and fixed sections in a saturated solution of copper acetate at 40° for 24 hours and then washing them in a strong solution of sodium carbonate. A general reduction of the copper occurred in the fresh sections, but the individual cells could not be studied. In the fixed films the only cells which reduced were those of the rete malpighii. Therefore, although we can say that the tissue cells do contain sugar, we cannot differentiate them individually.

We therefore resorted to a physical test.

By the use of Nicol's prisms it is seen that the syphilitic cells when properly focussed appear as bright stars against a black background. The phenomenon is better marked in specimens fixed with absolute alcohol than when 50 % alcohol has been used for the same purpose, and can be beautifully demonstrated in sections stained with pyronin and methyl green. It is most marked over the nuclear area, is greater in zygotes than in female gametocytes, is very well marked in the trophozoite and male gametocyte and much less evident in sporoblasts and sporozoites. In short, it is greatest where the pyronin staining is deepest; therefore, it is the pyroninophile substance that is concerned. The only cell constituents which exhibit this property are cholesterol, sugar, and lipoids.

Cholesterol gives a crystalline, and not a star-like appearance as seen in the syphilitic parasites, and moreover cholesterol is not increased in the serum of syphilitic patients. Sugar can be excluded, because if it were present in sufficient quantities to show the phenomenon, it would give the micro-chemical tests; moreover sugar is soluble in water which the pyroninophile substance is not. Further, the active substance of the parasitic cells must therefore be lecithin, or rather its fatty acid constituent. To bring still greater evidence to bear on this assumption we compared the colloidal particles in a case of

pseudochylous fluid with dextrose and in a case without dextrose, with the result that the former were more active than the latter, the activity of which was comparable in degree with that of the syphilitic cells. The pseudochylous fluid with dextrose gave a marked Berlin blue reaction, which was not the case with the other. The fluid containing no dextrose had a reducing action equal to that of the syphilitic cells; therefore it appears justifiable to state that the syphilitic bodies contain no dextrose.

Leaving sections for hours and even days in ether, absolute alcohol, and absolute alcohol and ether mixed, the cells still retain this property and therefore the lecithin cannot exist alone. The pyroninophile substance contains protein, and as a lecithin-protein complex is known to exist and the protein is a globulin which we have also shown the pyroninophile substance to be, we can assume that this substance is lecithin-globulin.

Fatty acids. Now what evidence have we for assuming that it contains a fatty acid? We stained sections with iodine and with Nile blue sulphate, according to the method of Lorrain Smith. The syphilitic bodies stained with iodine and also very deeply with a saturated solution of Nile blue sulphate. Red blood corpuscles also stained with iodine and all nuclei and nucleoli stained with Nile blue sulphate. The only granoplasma that stained with Nile blue sulphate was that of the syphilitic bodies and some of the plasma cells. The inclusion of a fatty acid in the lecithin-globulin complex is still further supported by the invariable occurrence of a saturated fatty acid (stearic acid) in the lecithin-globulin complex of pseudochylous fluids, and the visibility of the fluid between crossed Nicol prisms is due to the fatty acid and not to the lecithin itself.

The reducing action of red blood corpuscles, syphilitic bodies, protoplasm of some plasma cells and nucleoli is in all probability due to the lecithin-globulin complex and the change that takes place in impregnated female cells is an increase of this body which, owing to its reducing action, prevents the cell from staining with methyl green, and, owing to its acid action, prevents the cell from showing a marked affinity for negatively charged dyes, as carbol fuchsin, etc. Since the complex is not so marked in unimpregnated female cells and the protein is less acid, it will stain with carbol fuchsin in the carbol fuchsin-carbol iodine green method. Owing to the staining reactions, the fatty acid must be a saturated one, and as neither the syphilitic bodies nor the colloidal particles of pseudochylous fluid stain with osmic acid or Sudan III, the fatty acid is clearly not oleic acid.

As the syphilitic parasite was shown to contain lecithin we thought it necessary to repeat the more important work which had been done in

connection with the lecithin in nerve tissue, so the following tests were carried out.

1. The tissue was fixed in Müller's fluid for 10 days, frozen and sections cut. The sections were transferred into 1 % osmic acid for 24 hours at 37° and then placed in the following mixture:—

Pyrogallic acid	...	...	parts 15
Sod. sulphite	...	...	,, 125
Sod. nitrate ...	...	...	,, 70
Water ...	...	...	,, 300

and differentiated in 0·1 % potassium permanganate which reoxidises the osmium which has not combined. The brown colour of the permanganate can be removed if desired with 1 % oxalic acid.

Considering how insoluble the syphilitic lecithin appeared to be in alcohol, we tried alcohol fixed specimens and paraffin sections, but instead of floating the cut sections out on to warm water, we employed a hot 7 % solution of potassium bichromate and obtained quite as good results. The syphilitic bodies had a greater reducing action upon the osmium than other cells, staining a deep olive green, the only other structures which resembled them were nucleoli and the aminoplasma cells.

2. Tissues were fixed for four days in 10 % formalin and were then placed for the same length of time in Weigert's chrome alum copper acetate mixture in the incubator at 37°. Some sections were cut in the frozen state, others taken through paraffin. The cut sections were put into sulphuric acid alcohol (1 : 500 H_2SO_4 in 50 % alcohol), and then stained for 10 minutes in 1 % osmic acid; they were then well washed and treated with 5 % pyrogallic acid solution, differentiated in 0·1 % potassium permanganate taken through sulphurous acid and the fresh sections were mounted in liquid paraffin; while the paraffin sections went through the usual stages to balsam. The results tallied with those obtained from No. 1.

3. Alcohol fixed specimens, the paraffin sections of which had been floated out on to potassium bichromate, were stained for 24 hours at room temperature in Weigert's haematoxylin.

The syphilitic bodies stain intensely and in this respect resemble the nucleoli. The chromatin of nuclei also stains well, but the colour is not so fast.

4. Paraffin sections of tissue which had been floated out on potassium bichromate were stained for 24 hours at 37° in Kultschitzky's haematoxylin, washed and decolourised in a 0·25 % solution of sodium carbonate until the sections retained a light blue colour. The sections were then washed,

transferred to a 0·1 % solution of potassium permanganate, then into sulphurous acid, washed in a 0·1 % solution of lithium carbonate, taken through alcohol and mounted in balsam.

The sections gave the same results as were obtained from method No. 3.

All sections were compared with and controlled by sections from a case of neurofibromatosis, with the result that the reducing action of the syphilitic bodies was practically equal to that of the medullated nerve fibres, and the capacity for staining with haematoxylin resembled that of the axis cylinders, the deep colour of which is no doubt due to the presence of a lecithin-protein complex.

Summary. The reducing action of the pyroninophile substance of the syphilitic bodies is not due to cholesterol or a carbohydrate. Although it is active optically, it is not so markedly so as the two substances just mentioned.

The activity is due to a fatty acid and resembles that of the particles met with in the fluid from a case of pseudochylous ascites. The particles of such a fluid consist of lecithin-globulin with a saturated fatty acid (stearic acid) in its radicle. This physical phenomenon is a most useful means of picking out the syphilitic bodies in a section.

Oxidising enzymes.

Our next efforts were to ascertain whether the presence of an oxydase or a peroxydase could be detected in the syphilitic bodies, since some observers have stated that the bodies which have been described as parasitic are really mast cells. For this purpose we fixed some tissue in formalin embedded in gelatin, and cut sections after freezing the material with carbon dioxide.

In testing for oxydases, the following methods were employed:—

1. Sections were placed in a 1 % alcoholic solution of benzidine, but failed to stain, proving that no oxydase could be demonstrated; therefore, the syphilitic bodies do not harbour a tyrosinase, a ferment which at first sight might be expected to be present, owing to the increased pigmentation which is somewhat characteristic of syphilitic lesions.

2. Sections were placed in a 1 % alkaline solution of α-naphthol (prepared by heating 1 grm. α-naphthol in 100 cc. dist. water and then adding just sufficient KOH to bring the α-naphthol into solution) for a few minutes and then transferred to a 1 % sol. of tetramethylparaphenylene-diamine hydrochloride (violamine) washed in distilled water and mounted in glycerol or liquid paraffin. This method also failed to show the presence of an oxydase.

The following methods used for demonstrating the presence of a peroxydase met with more success:—

1. Sections were placed in a mixture of equal parts of a 1 % alcoholic solution of benzidine and 3 % hydrogen peroxide.

Such sections can be taken through alcohol, etc. and mounted in balsam, or of course mounted in the ordinary way. A very strong peroxydase reaction was found in the red blood corpuscles and mast cell granules. The syphilitic bodies remained unstained.

It is owing to the presence of peroxydases that the mast cells stain orange with pyronin.

2. Equally good results can be obtained by leaving sections in a mixture of paraphenylenediamine tartrate (ursoltartrate) and H_2O_2.

The presence of a peroxide in tissues cannot be demonstrated, as no blue colour results on leaving sections in a mixture of benzidine and extract of horseradish. The above affords sufficient evidence against the view that the syphilitic bodies are mast cells.

Physical and chemical properties of the lecithin-globulin complex.

The lecithin-globulin complex when in solution gives rise to a definite opalescence, and exists as a colloidal suspension which can be removed by filtration through a Chamberland candle. It appears microscopically in the form of very fine refractile granules which do not stain with osmic acid, or Sudan III. These fine granules no doubt owe their refractility to the associated lipoid lecithin. The particles exist in an alkaline medium and possess a negative charge, and in consequence we find that the lecithin-globulin complex is readily precipitated by kations, particularly the divalent kations, Mg, Ba, and Ca.

The lecithin-globulin compound is readily precipitated by acetic acid even in the cold, also by alcohol; half saturation or full saturation with ammonium sulphate, and removal of the salts by dialysis results in separation of the lecithin-globulin complex. Treatment of the complex with ether has no effect and previous addition of alkali such as caustic potash does not appreciably alter the solubility of this body. In all respects this lecithin-globulin behaves exactly like the pseudo-globulin fraction isolated from serum. One third saturation with $(NH_4)_2SO_4$ precipitates the euglobulin fraction from serum and this fraction is soluble in a 0·6 % solution of sodium chloride,

whereas the pseudo-globulin remains insoluble. The pseudo-globulin fraction of the serum thus behaves in every way like the lecithin-globulin compound. The solubility of the globulin present in serum or in the body cells will therefore depend upon the amount of lipoid in association with the globulin, and the former will influence the optical properties, the electrical charge on the colloidal particles in suspension, and also the relationship of globulin to electrolytes. In connection with these observations, it may be noted how important both constituents of the complex are in the maintenance of life. During starvation, for example, the blood contains a larger amount of globulin, and after excessive bleeding the first constituent of the blood to return to its normal amount is the globulin fraction.

With regard to the nature of the lipoid present in association with the globulin, it is usually found to be lecithin. This lecithin is generally of the type described as a monoaminophosphatide, yielding choline on hydrolysis, and fatty acids of the stearyl group. The lecithin is insoluble in water, and is so firmly united to the globulin as to remain undissolved when treated with ether. The production of lecithin is probably determined by the processes of degeneration of cellular material, which takes place in diseases in which effusions may result, viz. tuberculous infections, malignant disease, and syphilis. In the production of milky effusions it seems highly probable that the destruction of lecithin-containing cell elements takes place in the blood itself, and that the lecithin so formed diffuses through the peritoneal membrane into the serous cavities. This would explain the sudden changes from a clear effusion to a milky one noted by some observers, or the reverse condition where a milky is later replaced by a clear transparent fluid.

The resistance to putrefaction exhibited by all fluids containing this complex is very striking in view of the fact that lecithin readily undergoes auto-oxidation when free. The complex seems to confer increased stability upon both constituents, but at the same time the lecithin is capable of fully exerting all its special functions, particularly its influence on the neutralisation of toxines and bacterial growth. The chemical configuration of the lecithin molecule possibly accounts for this property, since it contains a large number of hydroxyl groups capable of uniting with such bodies as ferments, proteins, sugar, and other lipoids. The power lecithin possesses of resisting putrefaction suggests a possible function of this body in the production of immunity.

From what has been stated it will be noticed how close is the resemblance between the pyroninophile substance of the syphilitic parasite and the colloidal particles of the pseudochylous fluid, both substances being no doubt

identical and the relationship becomes the closer when the staining properties of both are considered, and for brevity's sake we need only state that both the colloidal particles and the syphilitic parasites are Gram negative.

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NOTE:

CHICK & MARTIN. *The Precipitation of egg-albumin by ammonium sulphate.* This Journal, VII, 1913, p. 380. In Table VIII, p. 392, 4th column, the values given for the percentage by weight in the Pressed Precipitate of $(NH_4)_2SO_4$ and water respectively should be interchanged and the Table should read thus:

Exp.	Composition, % by weight of	In pressed precipitate
I	$(NH_4)_2SO_4$	6·39
	H_2O	29·83
II	$(NH_4)_2SO_4$	8·47
	H_2O	29·91
III	$(NH_4)_2SO_4$	6·39
	H_2O	22·04